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<b>(21) International Application Number:</b> PCT/US96/05798 <b>(22) International Filing Date:</b> 26 April 1996 (26.04.96)  <b>(30) Priority Data:</b> 08/430,908 27 April 1995 (27.04.95) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/430,908 (CIP) Filed on 27 April 1995 (27.04.95)  <b>(71) Applicant (for all designated States except US):</b> AFFYMAX TECHNOLOGIES N.V. [NL/NL]; De Ruyderkade 62, Curacao (AN).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BARUCH, Dror, I. [IL/US]; 777 San Antonio Road #123, Palo Alto, CA 94303 (US). PASLOSKE, Brittan, L. [CA/US]; 501 Carter Drive, Austin, TX 78740 (US). HOWARD, Russell, J. [AU/US]; 12700 Viscaio Road, Los Altos, CA 94022 (US).		<b>(74) Agents:</b> MURPHY, Matthew, B. et al.; Townsend and Townsend and Crew, 20th floor, One Market Plaza, Steuart Street Tower, San Francisco, CA 94105 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
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<b>(57) Abstract</b>  The present invention generally relates to novel proteins, and fragments thereof, as well as nucleic acids which encode these proteins, and methods of making and using these proteins in both diagnostic and therapeutic applications. In particular, the present invention relates to PfEMP1 proteins and fragments thereof which are associated with the pathology of malaria infections, and which may be used in preventing, diagnosing and/or treating the symptoms of patients who suffer from malaria and associated diseases.		

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## MALARIA PEPTIDES AND VACCINES

5           This application is a continuation-in-part of U.S. Patent Application Serial No. 08/430,908, filed April 27, 1995, which is hereby incorporated herein by reference in its entirety for all purposes.

10           The present invention generally relates to novel proteins, and fragments thereof, as well as nucleic acids which encode these proteins, and methods of making and using these proteins in diagnostic, prophylactic and therapeutic applications. In particular, the present invention relates to proteins from the *Plasmodium falciparum* erythrocyte membrane  
15           protein 1 ("PfEMP1") gene family and fragments thereof which are derived from malaria parasitized erythrocytes. In particular, these proteins are derived from the erythrocyte membrane protein of *Plasmodium falciparum* parasitized erythrocytes, also termed "PfEMP1". The present invention  
20           also provides nucleic acids encoding these proteins, which proteins and nucleic acids are associated with the pathology of malaria infections, and which may be used as vaccines or other prophylactic treatments for the prevention of malaria infections, and/or in diagnosing and treating the symptoms of  
25           patients who suffer from malaria and associated diseases.

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### BACKGROUND OF THE INVENTION

30           Erythrocytes infected with the malaria parasite *P. falciparum* disappear from the peripheral circulation as they mature from the ring stage to trophozoites (Bignami and Bastianelli, *R forma Medica* (1889) 6:1334-1335). This  
35           phenomenon, known as sequestration, results from parasitized erythrocyte ("PE") adherence to microvascular endothelial cells in diverse organs (Miller, *Am. J. Trop. Med. Hyg.* (1969)

18:860-865). Sequestration is associated temporally with expression of knob protrusions (Leech et al., *J. Cell. Biol.* (1984) 98:1256-1264), expression of a very large antigenically variant surface protein, called PfEMP1 (Aley et al., *J. Exp. Med.* (1984) 160:1585-1590; Leech et al., *J. Exp. Med.* (1984) 159:1567-1575; Howard et al., *Molec. Biochem. Parasitol.* (1988) 27:207-223), and expression of new receptor properties which mediate adherence to endothelial cells (Miller, *supra*; Udeinya et al., *Science* (1981) 213:555-557. Endothelial cell surface proteins such as CD36, thrombospondin (TSP) and ICAM-1 have been identified as major host receptors for mature PE. See, e.g., Barnwell et al., *J. Immunol.* (1985) 135:3494-3497; Roberts et al., *Nature* (1985) 318:64-66; and Berendt et al., *Nature* (1989) 341:57-59.

PE sequestration confers unique advantages for *P. falciparum* parasites (Howard and Gilladoga, *Blood* (1989) 74:2603-2618), but also contributes directly to the acute pathology of *P. falciparum* (Miller et al., *Science* (1994) 264:1878-1883). Of the four human malaras, only *P. falciparum* infection is associated with neurological impairment and cerebral pathology seen increasingly in severe drug-resistant malaria (Howard and Gilladoga, *supra*). Although the genesis of human cerebral malaria is likely due to a combination of factors including particular parasite phenotypes (Berendt et al., *Parasitol. Today* (1994) 10:412-414), inappropriate immune responses and the phenotype of endothelial cell surface molecules in the cerebral microvasculature (Pasloske and Howard, *Ann. Rev. Med.* (1994) 45:283-295), adherence of PE to cerebral blood vessels and consequent local microvascular occlusion is a major contributing factor. See, e.g., Berendt et al., *supra*; Patnaik et al., *Am. J. Trop. Med. Hyg.* (1994) 51:642-647.

The capacity of *P. falciparum* PE to express variant forms of PfEMP1 contributes to the special virulence of this parasite. Variant parasites can evade variant-specific antibodies elicited by earlier infections. The *P. falciparum* variant antigens have been defined in vitro using antiserum prepared in Aotus monkeys infected with individual parasite



strains (Howard et al., *Molec. Biochem. Parasitol.* (1988) 27:207-223). Antibodies raised against a particular parasite will only react by PE agglutination, indirect immuno-fluorescence or immunoelectronmicroscopy with PE from the same strain (van Schravendijk et al., *Blood* (1991) 78:226-236). Such studies with PE from malaria patients in diverse geographic locations and sera from the same or different patients confirm that PE in natural isolates express variant surface antigens and that individual patients respond to infection by production of isolate-specific antibodies (Marsh and Howard, *Science* (1986) 231:150-153; Aguiar et al., *Am. J. Trop. Med. Hyg.* (1992) 47:621-632; Iqbal et al., *Trans. R. Soc. Trop. Med. Hyg.* (1993) 87:583-588. Expression of a variant antigen on PE has also been demonstrated in several simian, murine and human malaria species, including *P. knowlesi* (Brown and Brown, *Nature* (1965) 208:1286-1288; Barnwell et al., *Infect. Immun.* (1983) 40:985-994), *P. chabaudi* (Gilks et al., *Parasite Immunol.* (1990) 12:45-64; Brannan et al., *Proc. R. Soc. Lond. Biol. Sci.* (1994) 256:71-75), *P. fragile* (Handunnetti et al., *J. Exp. Med.* (1987) 165:1269-1283) and *P. vivax* (Mendis et al., *Am. J. Trop. Med. Hyg.* (1988) 38:42-46). Laboratory studies with *P. knowlesi* (Brown and Brown, *supra*; Barnwell et al., *supra*) or *P. falciparum* (Hommel et al., *J. Exp. Med.* (1983) 157:1137-1148) in monkeys and *P. chabaudi* in mice (Gilks et al., *supra*) confirmed that antigenic variation at the PE surface is associated with prolonged or chronic infection and the capacity to repeatedly re-establish blood infection in previously infected animals. Studies with cloned parasites demonstrated that antigenic variants can arise with extraordinary frequency, e.g., 2 % per generation with *P. falciparum* (Roberts et al., *Nature* (1992) 357:689-692) and 1.6 % per generation with *P. chabaudi* (Brannan et al., *supra*).

PfEMP1 was identified as a <sup>125</sup>I-labeled, size diverse protein (200-350 kD) on PE that is lacking from uninfected erythrocytes, and that is also labeled by biosynthetic incorporation of radiolabeled amino acids (Leech et al., *J. Exp. Med.* (1984) 159:1567-1575; Howard et al.,

Molec. Biochem. Parasitol. (1988) 27:207-223). PfEMP1 is not extracted from PE by neutral detergents such as Triton X-100 but is extracted by SDS, suggesting that it is linked to the erythrocyte cytoskeleton (Aley et al., *J. Med. Exp.* (1984) 160:1585-1590). After addition of excess Triton X-100, PfEMP1 is immunoreactive with appropriate serum antibodies (Howard et al., (1988), *supra*). Mild trypsinization of intact PE rapidly cleaves PfEMP1 from the cell surface (Leech et al., *J. Exp. Med.* (1984) 159:1567-1575). PfEMP1 bears antigenically diverse epitopes since it is immunoprecipitated from particular strains of *P. falciparum* by antibodies from sera of Aotus monkeys infected with the same strain, but not by antibodies from animals infected with heterologous strains (Howard et al. (1988), *supra*). Knobless PE derived from parasite passage in splenectomized Aotus monkeys (Aley et al., *supra*) do not express surface PfEMP1 and are not agglutinated with sera from immune individuals or infected monkeys (Howard et al. (1988), *supra*; Howard and Gilladoga, *Blood* (1989) 74:2603-2618). In general, sera that react with the PE surface by indirect immunofluorescence and antibody-mediated PE agglutination are the only sera to immunoprecipitate <sup>125</sup>I-labeled PfEMP1 from any particular strain (Howard et al., (1988), *supra*; van Schravendijk et al., *Blood* (1991) 78:226-236; Biggs et al., *J. Immunol.* (1992) 149:2047-2054).

The adherence of parasitized erythrocytes to endothelial cells is mediated by multiple receptor/counter-receptor interactions, including CD36, thrombospondin and intracellular adhesion molecule-1 (ICAM<sub>1</sub>) as the major host cell receptors (Howard and Gilladoga, *Blood* (1989) 74:2603-2618, Pasloske and Howard, *Ann. Rev. Med.* (1994) 45:283-295). Vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1) have also been implicated as additional endothelial cell receptors that can mediate adherence of a minority of *P. falciparum* PE (Ockenhouse, et al., *J. Exp. Med.* (1992) 176:1183-1189, and Howard and Pasloske, *supra*). The adherence receptors on the surface of PE has not yet been conclusively identified, and several molecules, including AG 332 (Udomsangpetch, et al.,

Nature (1989) 338:763-765), modified band 3 (Crandall, et al., Proc. Nat'l Acad. Sci. USA (1993) 90:4703-4707), Sequestrin (Ockenhouse, Proc. Nat'l Acad. Sci. USA (1991) 88:3175-3179), and PfEMP1 (Howard and Gilladoga, supra, and Pasloske and Howard, supra), have been proposed as candidates. Several pieces of indirect evidence have linked expression of PfEMP1 with the acquisition of new host protein receptor properties on the surface of PE (Howard and Gilladoga, supra; Pasloske and Howard, Ann. Rev. Med. (1994) 45:283-295). PE adherence is correlated with the expression of PfEMP1 on the surface of mature stage PE (Leech, et al., J. Exp. Med. (1984) 159:1567-1575). Alterations in the adherence phenotype of the PE selected for *in vitro* are usually associated with the emergence of new forms of PfEMP1 (Biggs, et al., J. Immunol. (1992) 149:2047-2054; Roberts, et al., Nature (1992) 357:689-692). Mild trypsinization of intact mature PE cleaves the extracellular portion of PfEMP1 and at the same time, reduces or eliminates PE cytoadherence (Leech, et al., supra). Previously described antibody mediated blockade or reversal of cytoadherence is strain specific and is correlated with the ability of the reacting sera to agglutinate the corresponding PE and to immunoprecipitate the surface labeled <sup>125</sup>I-PfEMP1 (Howard, et al., Molec. Biochem. Parasitol. (1988) 27:207-224). Pfalhesin (modified band 3) have been shown to bind CD36 under non-physiological conditions (Crandall, et al., Exp. Parasitol. (1994) 78:203-209). Sequestrin, which appears to be homologous to PfEMP1, extracted with TX100 from knobless PE, was shown to bind to immobilized CD36 (Ockenhouse, Proc. Nat'l Acad. Sci. USA (1991) 88:3175-3179).

The complex nature and/or mechanism of malarial antigenic variation, and its particular virulence has created a need for methods and compositions which may be useful in the treatment diagnosis and prevention of malaria infections. The present invention meets these and other needs.

#### SUMMARY OF THE INVENTION

It is a general object of the invention to provide proteins and polypeptides that are derived from PfEMP1

proteins, nucleic acids encoding these proteins and antibodies that are specifically immunoreactive with these proteins. It is a further object to provide methods of using these various compositions in diagnosis, treatment or prevention of the onset of symptoms of a malaria parasite infection. It is a further object to provide methods of screening compounds to identify further compositions which may be used in these methods.

In one embodiment, the present invention provides substantially pure polypeptides which have amino acid sequences substantially homologous to the amino acid sequence of a PfEMP1 protein, or biologically active fragments thereof. In preferred aspects, the polypeptides of the present invention are substantially homologous to the amino acid sequence shown in Figure 2 or 12, biologically active fragments or analogues thereof. Also provided are pharmaceutical compositions comprising these polypeptides.

In another embodiment, the present invention provides nucleic acids which encode the above described polypeptides. Particularly preferred nucleic acids will be substantially homologous to a part or whole of the nucleic acid sequence shown in Figure 12 or the nucleic acid encoding for the sequences shown in Figures 20 or 21. The present invention also provides expression vectors comprising these nucleic acid sequences and cells capable of expressing same.

In an additional embodiment, the present invention provides antibodies which recognize and bind PfEMP1 polypeptides or biologically active fragments thereof. More preferred are those peptides which recognize and bind PfEMP1 proteins associated with infection by more than one variant of *P. falciparum*.

In a further embodiment, the present invention provides methods of inhibiting the formation of PfEMP1/ligand complex, comprising contacting PfEMP1 or its ligands with polypeptides of the present invention.

In a related embodiment, the present invention provides methods of inhibiting sequestration of erythrocytes in a patient suffering from a malaria infection, comprising

administering to said patient, an effective amount of a polypeptide of the present invention. Such administration may be carried out prior to or following infection.

5 In still another embodiment, the present invention provides a method of detecting the presence or absence of PfEMP1 in a sample. The method comprises exposing the sample to an antibody of the invention, and detecting binding, if any, between the antibody and a component of the sample.

10 In an additional embodiment, the present invention provides a method of determining whether a test compound is an antagonist of PfEMP1/ligand complex formation. The method comprises incubating the test compound with PfEMP1 or a biologically active fragment thereof, and its ligand, under conditions which permit the formation of the complex. The  
15 amount of complex formed in the presence of the test compound is determined and compared with the amount of complex formed in the absence of the test compound. A decrease in the amount of complex formed in the presence of the test compound is indicative that the compound is an antagonist of PfEMP1/ligand  
20 complex formation.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a map of two Malayan Camp strain ("MC") PfEMP1 genes and recombinant protein fragments ("RP").  
25 The predicted open reading frame is shown starting from nucleotide +1. CDNA clones A1 through E1 and G1 are located with their boundaries (nucleotide number). Each clone was shown by PCR to be physically linked to the adjacent clones and confirmed by sequence overlap. Clone D3 was linked 3' only to  
30 clone E1 and not to clones D1 or D2. gDNA clone F-gDNA was linked by sequence overlap to cDNAs D1 and D2. The regions of PfEMP1 expressed as GST fusion proteins, rB1, rC1-1, rC1-2, rD1, are shown, with the total amino acid length of each and boundary amino acids.

35 Figure 2 shows the predicted amino acid sequence of two MC PfEMP1 genes deduced from cDNA and gDNA clones, up to amino acid 2924. The position of the putative 725 bp intron (nucleotides 7429-8153) is indicated by a vertical arrow. The

likely transmembrane domain is boxed. After amino acid 871 the extensive sequence differences in cDNAs D2 and D3 are shown as separate sequences extending 3' with contiguity to F-gDNA and cDNA E1 respectively. Amino acid sequence identity in these two sequences is shown in bold. The four Duffy Binding Ligand ("DBL") domains, denoted DBL-1 through DBL-4 and three cysteine-rich motifs ("CRM") between the DBL domains denoted CRM-1 through CRM-3, are shaded. Consensus amino acids in each DBL domain are underlined and conserved cysteines of the CRM motif are indicated by underlined dots.

Figure 3 shows alignment of the three CRMs with amino acid numbers indicated. CRM-1 and CRM-2 share the motif CX<sub>3</sub>CX<sub>3</sub>CXC. CRM-3 has less homology, that is more pronounced within a restricted sequence 2371-2390 that includes the CX<sub>3</sub>CXC motif.

Figure 4 shows autoradiographs of Southern blot hybridization of cDNA clones from the MC PfEMP1 gene with DNA from various *P. falciparum* parasites digested with Eco RI or Eco RI and Hind III. Panel A shows probing with cDNA A1, from the 5' end of the gene, shows hybridization to multiple bands with all *P. falciparum* parasites tested. Panel B shows probing with clone C1 showing hybridization to fewer bands with MC K+ and MC K- parasites only. Markers of molecular mass in kd are indicated on the left. Table 2 summarizes the results obtained with additional cDNA and gDNA probes.

Figures 5A-5E show immunoprecipitation of <sup>125</sup>I-PfEMP1 from MC K+ PE with non-crossreacting antibodies elicited by immunization with recombinant proteins. In Figures 5A and 5B, results with preimmune serum are shown on the left gel lane with results for post-immunization serum from the same animal on the right. Sera from rabbits (rab 1-6) and rats (rat 1-4) were used for immunoprecipitation of SDS extracts from MC K+ PE that had been surface labeled by lactoperoxidase catalyzed radioiodination. Immunoprecipitation was followed by SDS-PAGE and autoradiography. The markers of molecular mass in kiloDalton are indicated on the left. In Figure 5A, rabbits and rats were immunized with rC1-2. <sup>125</sup>I-PfEMP1 is identified on the left by immunoprecipitation with a strain-specific

anti-MC K+ serum. In Figure 5B, sera from two rabbits immunized with rD1. In Figure 5C, the  $^{125}\text{I}$ -band immunoprecipitated by anti-rC1-2 and anti-rD1 sera shares properties of detergent extraction and trypsin sensitivity with PfEMP1. MC K+ PE were radioiodinated and some of the cells treated with trypsin (5 min., 10  $\mu\text{g/ml}$ ). Sequential Triton X100 and SDS extracts were immunoprecipitated with three sera that define  $^{125}\text{I}$ -PfEMP1 of MC K+ parasites: pool of human immune serum; Aotus anti-MC K+ serum; rabbit 05-75 anti-PfEMP3 and PfEMP1 serum. The prebleed and post-rC1-2 immunization bleed from rabbit 1 were analyzed in parallel. In Figures 5D and 5E, the anti-PfEMP1 antibodies in anti-rC1-2 and anti-rD1 sera do not crossreact. Sera were preadsorbed with glutathione-Sepharose beads (none), with GST or GST fusion proteins derived from MC PfEMP1 (rB1, rC1-2, rD1) or other *P. falciparum* genes (rA62-5, rPfEMP3) and used for immunoprecipitation. Only a portion of the autoradiograph is shown.

Figure 6 shows the immunoblotting of diverse *P. falciparum* parasites with rabbit anti-rC1-2 serum identifying antigenic cross reactivity between the PfEMP1 protein of MC K+ parasites and PfEMP1 bands of several other parasites known to express antigenically distinct PfEMP1 antigens. SDS extracts from  $2.5 \times 10^5$  parasites (trophozoite and schizont stage) were subjected to SDS-PAGE and transferred to PVDF membrane. The membrane was incubated with rabbit #2 anti-rC1-2 serum, 1 hour at room temperature. Bound antibodies were visualized by the ECL western blot method.

Figure 7 shows antisera raised against the rC1-2 fragment of MC K+ PfEMP1 reacting with the surface of MC K+ in a strain-specific manner. Results shown for anti-rC1-2 rat serum #1. Panels A and B show indirect immunofluorescence of intact non-fixed PE of MC K+ strain detected by confocal fluorescence imaging microscopy. Cells (4% parasitemia) were incubated with anti-rC1-2 serum and visualized by TRITC-conjugated goat anti-rat IgG. Panel A is a bright field showing a pigmented (mature) PE and several uninfected erythrocytes. Panel B shows fluorescence of the same field

with reactivity only on the surface of the PE. The focal concentration of fluorescence is attributed to the narrow plane of confocal microscopy. The bar equals 10  $\mu$ m.

Figure 8 shows antibody mediated PE agglutination observed by light microscopy. Anti-rC1-2 serum agglutinated mature MC K+ PE (1:20 dilution) but not MC K- PE or K+C+ ItG2-ICAM PE (1:5 dilution). Pre-immune (prebleed) serum of the same animal did not agglutinate MC K+ PE (1:5 dilution). Aotus anti MC K+ sera only agglutinated MC K+ PE. The bar equals 500  $\mu$ m. The infected blood showed 8-15% parasitemia. Similar results obtained with other anti-rC1-2 sera are summarized in Table 3.

Figure 9 shows immunoelectron-microscopy of intact MC K+ PE with anti-rC1-2 serum identified PfEMP1 expression specifically at knob protrusions rather than at areas of the PE surface membrane between knobs. Treatment with rat antiserum was followed by treatment with 5 nm gold-conjugated goat anti-rat IgG. 5 nm gold particles were deposited on >50% of the knobs.

Figure 10 shows that antisera to the rC1-2 fragment of MC K+ PfEMP1 block adherence of MC K+ PE to immobilized CD36 but do not block adherence to immobilized TSP. PE (or PRBC) were preincubated with diluted serum before transfer to wells containing the immobilized proteins. The number of adherent PE remaining after washing was determined by light microscopy counting. Sera collected at day 28, after 2 immunizations was also active. Results shown as means and standard deviations of quadruplicate assays. The graph shows PE preincubated with binding medium (BM) alone (no addition) or with BM containing 1:5 dilution or rat anti-PfEMP3 serum or rat anti-rC1-2 sera (rat #1-4). Rat anti-rC1-2 sera blocked adherence of PE to CD36 (solid bars) but not to TSP (hatched bars). The inhibition of adherence observed with sera from rats 1, 2, and 4 were significantly different from that of the control ( $p < 0.0004$ ).

Figure 11 shows the concentration dependent blockade of adherence to CD36 with rat anti-rC1-2. Immune (filled circles) and pre-immune serum (open circles) from rat #1 were



tested at different dilutions for blockade of adherence of MC K+ PE to CD36.

Figures 12A-12L show the nucleic acid sequence with the deduced amino acid sequence of the MC PfEMP1 gene. After  
5 base 2613 the extensive sequence differences in cDNAs D2 and D3 are shown as separate sequences extending 3' with contiguity to F-gDNA and cDNA E1 respectively.

Figure 13 shows the binding of CHO cells expressing surface CD36, to immobilized recombinant PfEMP1 protein  
10 fragments. CHO-CD36 cells were shown to bind to the fragment denoted rC1-2, however no binding was observed with the other fragments tested. Similarly no binding was observed with CHO-ICAM cells or control CHO cells.

Figure 14 shows the binding of CHO-CD36 cells in  
15 cells/mm<sup>2</sup> as a function of rC1-2 concentration added to the solid support.

Figures 15A and 15B are Western blots showing binding of CD36 to immobilized rC1-2. Figure 15A shows that CD36 binds to immobilized rC1-2 and not to GST or to the  
20 MCvar-1 recombinant proteins rA1(3-158), rB1(161-385), rC1-1(402-605), rD1(818-1003), rD2(982-1320), rF1-1(1300-1707), rF1-2(1688-2190), rF1-3(2171-2450), rG1(2550-2794) or the MCvar-2 specific recombinant proteins rD3(992-1243), rE1-1(1219-1471) and rE1-2(1454-1719) or to the RP fusion partner,  
25 GST. Figure 15B again shows that rC1-2 binds CD36, but fails to bind other cell surface receptors (P-selectin, L-selectin, E-selectin, VCAM-1 and ICAM-1).

Figure 16 shows a bar graph showing blockade of PE adherence to CD36 (shown as % of PE binding to immobilized  
30 CD36), in the presence of various fragments of PfEMP1, e.g., rA62-5, rB1, rC1-2 and rD1, and the GST fusion partner (rGST). Treatment with rC1-2 substantially blocks adherence of PE of different *P. falciparum* strains to CD36 (MC R+ (solid bars), clone ItG2-ICAM (hatched bars), clone ItG2-G1 (grey bars) and  
35 clon Palo Alto K- C+ (open bars)).

Figure 17 is a bar graph showing the effects of different fragments of PfEMP1 on the binding of Malayan Camp strain and ItG2-ICAM strain parasitized erythrocyte to CD36.

Figure 18 shows the binding of PE from MC and ItG-ICAM strains to CD36 as a function of rC1-2 concentration.

Figure 19 shows binding of CD36 to immobilized fragments of rC1-2. Shown is the binding to fragment rC1-2[1-233], rC1-2[1-59], rC1-2[1-87], rC1-2[1-102], rC1-2[1-140], rC1-2[1-192] and full length rC1-2. The fragment designations indicate the position of the starting and ending amino acids from amino acids 575-808 (or 1 through 233) of the sequence shown in Figure 2.

Figure 20 shows deduced amino acid sequences of the corresponding rC1-2[1-179] region of PfEMP1 genes from 11 different *P. falciparum* strains and clones. These sequences were obtained by PCR using the 1 and 179 primer set of MC PfEMP1. Sequences were obtained by amplification from genomic DNA (indicated by lower case g before the strain designation) by PCR from cDNA libraries (cMC and cFVO) or RT-PCR (cItG-F6) and cC5. These sequences, indicated by lower case c before the strain designation, represent the product of an expressed var gene. Conserved amino acids are indicated in the consensus sequence at the top of the sequence alignment chart.

Figure 21 shows the deduced amino acid sequences of the corresponding rC1-2[10-151] region of PfEMP1 genes from six different *P. falciparum* strains and clones. These sequences were obtained by PCR using the universal primer set deduced from the sequences shown in Figure 20. Sequences were obtained by amplification from genomic DNA (indicated by the small g before the name of the strain) and by PCR from cDNA libraries (cMC). These sequences, indicated by the small c, represent the product of an expressed var gene. In the MC strain, multiple var genes are shown. Conserved amino acids are indicated in the consensus sequence shown above the listed sequences.

Figure 22 shows the predicted structure of the MC PfEMP1 gene product. The figure shows the size and location of recombinant proteins (GST and MBP) derived from the sequence of MCvar-1 and MCvar-2.

Figure 23 shows immunoprecipitation with anti-rC1-2 and anti-MC specific sera and affinity purification with

immobilized CD36 and TSP of labeled fragments cleaved from the surface of iodinated PE of strain MC K+ by mild trypsinization.

5 Figure 24 shows the concentration dependent blockade of adherence of strain MC PE to CD36 with rC1-2[1-233] and rC1-2[1-179]. Determined IC<sub>50</sub> values are 1.2  $\mu$ M for rC1-2 and 0.78  $\mu$ M for rC1-2[1-179].

10 Figure 25 shows concentration dependent reversal of adherence of strain MC PE to CD36 with rC1-2[1-233] with an IC<sub>50</sub> value of approximately 0.5  $\mu$ M.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention generally relates to the *Plasmodium falciparum* erythrocyte membrane protein 1 ("PfEMP1"), nucleic acids which encode PfEMP1, and antibodies which specifically recognize PfEMP1. The polypeptides, antibodies and nucleic acids are useful in a variety of applications including therapeutic, prophylactic, including vaccination, diagnostic and screening applications.

20 The data described herein, indicates that PfEMP1 is responsible for both antigenic variation and receptor properties on PE, both of which are central to the special virulence and pathology of *P. falciparum*. The central role of PfEMP1 in *P. falciparum* biology, as the malarial adherence receptor for host proteins on microvascular endothelium, as described herein, indicates its usefulness in a malaria vaccine, in modelling prophylactic drugs, and also as a target for therapeutics to reverse PE adherence in acute cerebral malaria (Howard and Gilladoga, 1989).

30

#### I. Polypeptides of the Present Invention

Soluble PfEMP1 has been reported to bind to CD36, TSP and ICAM-1, and tryptic fragments of PfEMP1 cleaved from the PE surface have been shown to bind to TSP or CD36 (Baruch, et al., Molecular Parasitology Meeting at Woods Hole, Sept 18-22, 1994). Accordingly, in one aspect, the present invention provides substantially pur PfEMP1 polypeptides, analogs or biologically active fragments thereof.

35

The terms "substantially pure" or "isolated" refer, interchangeably, to proteins, polypeptides and nucleic acids which are separated from proteins or other contaminants with which they are naturally associated. A protein or polypeptide is considered substantially pure when that protein makes up greater than about 50% of the total protein content of the composition containing that protein, and typically, greater than about 60% of the total protein content. More typically, a substantially pure protein will make up from about 75 to about 90% of the total protein. Preferably, the protein will make up greater than about 90%, and more preferably, greater than about 95% of the total protein in the composition.

The term "biologically active fragment" as used herein, refers to portions of the proteins or polypeptides, e.g., a PfEMP1 derived polypeptide, which portions possess a particular biological activity, e.g., one or more activities found in a full length PfEMP1 polypeptide. For example, such biological activity may include the ability to bind a particular protein, substrate or ligand, to elicit antibodies reactive with PE, PfEMP1, the recombinant proteins or fragments thereof, to block, reverse or otherwise inhibit an interaction between two proteins, between an enzyme and its substrate, between an epitope and an antibody, or may include a particular catalytic activity. With regard to the polypeptides of the present invention, particularly preferred polypeptides or biologically active fragments include, e.g., polypeptides that possess one or more of the biological activities described above, such as the ability to bind a ligand of PfEMP1 or inhibit the binding of PfEMP1 to one or more of its ligands, e.g., CD36, TSP, ICAM-1, VCAM-1, ELAM-1, Chondroitin sulfate or by the presence within the polypeptide fragment of antigenic determinants which permit the raising of antibodies to that fragment.

The polypeptides of the present invention may also be characterized by their immunoreactivity with antibodies raised against PfEMP1 proteins or polypeptides. In particularly preferred aspects, the polypeptides are capable of inhibiting an interaction between a PfEMP1 protein and an

antibody raised against a PfEMP1 protein. Additionally or alternatively, such fragments may be specifically immunoreactive with an antibody raised against a PfEMP1 protein. Such fragments are also referred to herein as

5 "immunologically active fragments."

Generally, such biologically active fragments will be from about 5 to about 500 amino acids in length. Typically, these peptides will be from about 20 to about 250 amino acids in length, and preferably from about 50 to about

10 200 amino acids in length. Generally, the length of the fragment may depend, in part, upon the application for which the particular peptide is to be used. For example, for raising antibodies, the peptides may be of a shorter length, e.g., from about 5 to about 50 amino acids in length, whereas

15 for binding applications, the peptides may have a greater length, e.g., from about 50 to about 500 amino acids in length, preferably, from about 100 to about 250 amino acids in length, and more preferably, from about 100 to about 200 amino acids in length.

20 The polypeptides of the present invention may generally be prepared using recombinant or synthetic methods well known in the art. Recombinant techniques are generally described in Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, (2nd ed.) Vols. 1-3, Cold Spring Harbor Laboratory,

25 (1989). Techniques for the synthesis of polypeptides are generally described in Merrifield, *J. Amer. Chem. Soc.* 85:2149-2456 (1963), Atherton, et al., *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press (1989), and Merrifield, *Science* 232:341-347 (1986).

30 In preferred aspects, the polypeptides of the present invention may be expressed by a suitable host cell that has been transfected with a nucleic acid of the invention, as described in greater detail below.

Isolation and purification of the polypeptides of

35 the present invention can be carried out by methods that are generally well known in the art. For example, the polypeptides may be purified using readily available chromatographic methods, e.g., ion exchange, hydrophobic

interaction, HPLC or affinity chromatography, to achieve the desired purity. Affinity chromatography may be particularly attractive in allowing the investigator to take advantage of the specific biological activity of the desired peptide, e.g., ligand binding, presence of antigenic determinants, or the like.

Exemplary polypeptides of the present invention will generally comprise an amino acid sequence that is substantially homologous to the amino acid sequence of a PfEMP1 protein, or biologically active fragments thereof, or may include sequences that may take on a homologous conformation. In particularly preferred aspects, the polypeptides of the present invention will comprise an amino acid sequence that is substantially homologous to the amino acid sequence shown in Figure 2, Figure 12, Figure 20 and Figure 21, or a biologically active fragment thereof.

By "substantially homologous" is meant an amino acid sequence which is at least about 50% homologous to the amino acid sequence of PfEMP1 or a biologically active fragment thereof, preferably at least about 90% homologous, and more preferably at least about 95% homologous. In some aspects, substantially homologous may include a sequence that is at least 50% homologous, but that presents a homologous structure in three dimensions, i.e., includes a substantially similar surface charge or presentation of hydrophobic groups. Examples of preferred polypeptides include polypeptides having an amino acid sequence substantially homologous to the MC PfEMP1 amino acid sequence as shown in Figure 2 or Figure 12, and PfEMP1 of other *P. falciparum* strains as shown in Figures 20 and 21, as well as biologically active fragments of these polypeptides.

Preferred peptides include those peptide fragments of PfEMP1 that are involved in the sequestration of parasitized erythrocytes. Examples of these preferred peptides include peptides which comprise an amino acid sequence which is substantially homologous to amino acids 576 through 755 of the PfEMP1 amino acid sequence shown in

Figure 2 or Figure 12 or those sequences shown in Figures 20 and 21.

Also among the particularly preferred peptides of the present invention are those peptides and peptide fragments of PfEMP1 which are relatively conserved among the variant strains of *P. falciparum* or which contain regions of high homology to PfEMP1 proteins from other strains. The term "relatively conserved" generally refers to amino acid sequences that are substantially homologous to portions of the amino acid sequence shown in Figure 2 and Figure 12. However, also included within the definition of this term are peptides which are encoded by a nucleic acid which is a PCR product of primer probes, and particularly, universal primers, derived from the PfEMP1 nucleic acid sequence. In particular, primer probes derived from the nucleic acid sequence shown in Figure 12, may be used to amplify nucleic acids from other strains of *P. falciparum*. Particularly preferred primer sequences include the primer sequences shown in Table 1, below. Similarly, universal primer compositions, described in greater detail below and also shown in Table 1, may be used to amplify sequences that encode the peptides of the present invention.

Specific examples of relatively conserved peptides include those that are contained in a region of PfEMP1 proteins that corresponds to amino acids 576 through 755 of the amino acid sequence of MC PfEMP1, as shown in Figure 2. Similar regions have been specifically elucidated in a number of *P. falciparum* strains (See Figures 20 and 21). In general, these corresponding regions may be described as containing amino acid sequences that are encoded by the universal primer sequences described below. Generally, these amino acid sequences have one or more of the following general structures:



where  $X_1$  is selected from leucine or isoleucine,  $X_2$  is selected from glutamine and asparagine,  $X_3$  is selected from

the methionine, lysine and aspartic acid, X4 is selected from histidine, threonine and tyrosine and X5 is selected from aspartic acid, glutamic acid and histidine. In particularly preferred aspects, the polypeptides may contain both of the  
5 above general amino acid sequences. Particularly preferred amino acid sequences will possess the conserved amino acids shown in the various fragments shown in Figures 20 and 21. In particular, conserved amino acid sequences of six amino acids or greater, shown in Figures 20 and 21 (above the sequences),  
10 may be used as epitopes for generation of antibodies that cross react with multiple *P. falciparum* strains.

The peptides of the invention may be free or tethered, or may include labeled groups for detection of the presence of the polypeptides. Suitable labels include  
15 radioactive, fluorescent and catalytic labeling groups that are well known in the art and that are substantially described herein, e.g., signalling enzymes, chemical reporter groups, polypeptide signals, biotin and the like. Additionally, the peptides may include modifications to the N and C-termini of  
20 the peptide, e.g., an acylated N-terminus or amidated C-terminus.

Also included within the present invention are amino acid variants of the above described polypeptides. These variants may include insertions, deletions and substitutions  
25 with other amino acids. For example, in some aspects, amino acids may be substituted with different amino acids having similar structural characteristics, e.g., net charge, hydrophobicity, or the like. For example, phenylalanine may be substituted with tyrosine, as a similarly hydrophobic  
30 residue. Glycosylation modifications, either changed, increased amounts or decreased amounts, as well as other sequence modifications are also envisioned.

In addition to the above polypeptides which consist only of naturally-occurring amino acids, peptidomimetics of  
35 the polypeptides of the present invention are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide



compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) *Adv. Drug R s.* 15:29; Veber and Freidinger (1985) *TINS* p.392; and Evans et al. (1987) *J. Med. Chem* 30:1229, and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as naturally-occurring receptor-binding polypeptide, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of:  $-\text{CH}_2\text{NH}-$ ,  $-\text{CH}_2\text{S}-$ ,  $-\text{CH}_2-\text{CH}_2-$ ,  $-\text{CH}=\text{CH}-$  (cis and trans),  $-\text{COCH}_2-$ ,  $-\text{CH}(\text{OH})\text{CH}_2-$ , and  $-\text{CH}_2\text{SO}-$ , by methods known in the art and further described in the following references: Spatola, A.F. in *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins*, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A.F., *Vega Data* (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J.S., *Trends Pharm Sci* (1980) pp. 463-468 (general review); Hudson, D. et al., *Int J Pept Prot Res* (1979) 14:177-185 ( $-\text{CH}_2\text{NH}-$ ,  $\text{CH}_2\text{CH}_2-$ ); Spatola, A.F. et al., *Life Sci* (1986) 38:1243-1249 ( $-\text{CH}_2-\text{S}-$ ); Hann, M.M., *J Chem Soc Perkin Trans I* (1982) 307-314 ( $-\text{CH}-\text{CH}-$ , cis and trans); Almquist, R.G. et al., *J Med Chem* (1980) 23:1392-1398 ( $-\text{COCH}_2-$ ); Jennings-White, C. et al., *Tetrahedron Lett* (1982) 23:2533 ( $-\text{COCH}_2-$ ); Szelke, M. et al., *European Appln. EP 45665* (1982) CA: 97:39405 (1982) ( $-\text{CH}(\text{OH})\text{CH}_2-$ ); Holladay, M.W. et al., *Tetrahedron Lett* (1983) 24:4401-4404 ( $-\text{C}(\text{OH})\text{CH}_2-$ ); and Hruby, V.J., *Life Sci* (1982) 31:189-199 ( $-\text{CH}_2-\text{S}-$ ).

Peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent

attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the molecules to which the peptidomimetic binds (e.g., CD36) to produce the therapeutic effect.

Derivatization (e.g., labelling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic. Generally, peptidomimetics of peptides of the invention bind to their ligands (e.g., CD36) with high affinity and possess detectable biological activity (i.e., are agonistic or antagonistic to one or more ligand-mediated phenotypic changes).

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Gierasch (1992) *Ann. Rev. Biochem.* 61: 387; for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

Polypeptides of the present invention may also be characterized by their ability to bind antibodies raised against PfEMP1, or fragments thereof. Preferably, these antibodies recognize polypeptide domains that are homologous to the PfEMP1 proteins from a number of variants of *P. falciparum*. These homologous domains will generally be present throughout the family of PfEMP1 proteins. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein or domain. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay

formats and conditions that can be used to determine specific immunoreactivity. Antibodies to PfEMP1 and its fragments are discussed in greater detail, below. As used herein, the terms "polypeptide" or "peptide" are used interchangeably to refer to peptides, peptidomimetics, analogs, and the like, as described above.

The polypeptides of the present invention may be used as isolated polypeptides, or may exist as fusion proteins. A "fusion protein" generally refers to a composite protein made up of two or more separate, heterologous proteins which are normally not fused together as a single protein. Thus, a fusion protein may comprise a fusion of two or more heterologous or homologous sequences, provided these sequences are not normally fused together. Fusion proteins will generally be made by either recombinant nucleic acid methods, i.e., as a result of transcription and translation of a gene fusion comprising a segment encoding a polypeptide comprising a PfEMP1 protein and a segment which encodes one or more heterologous proteins, or by chemical synthesis methods well known in the art.

## II. Nucleic Acids of the Present Invention and Cells Capable of Expressing Same

Also provided in the present invention are isolated nucleic acid sequences which encode the above described polypeptides and biologically active fragments. Typically, such nucleic acid sequences will comprise a segment that is substantially homologous to a portion or fragment of the nucleic acid sequence shown in Figure 12, and more typically, the nucleic acid sequence from about nucleotide position -211 to about position 3559 of the nucleotide sequence shown in Figures 12, 20 and 21. Preferably, the nucleic acids of the present invention will comprise at least about 15 consecutive nucleotides of the nucleic acid sequence shown in Figures 12, 20 or 21, more preferably, at least about 20 contiguous nucleotides, still more preferably, at least about 30 contiguous nucleotides, and still more preferably, at least

about 50 contiguous nucleotides from the nucleotide sequence shown in Figures 12, 20 or 21.

Substantial homology in the nucleic acid context means that the segments, or their complementary strands, when compared, are the same when properly aligned with the appropriate nucleotide insertions or deletions, in at least about 60% of the nucleotides, typically, at least about 70%, more typically, at least about 80%, usually, at least about 90%, and more usually, at least about 95% to 98% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions to a strand, or its complement, typically using a sequence of at least about 15 contiguous nucleotides derived from the PfEMP1 nucleic acid sequence. However, larger segments will usually be preferred, e.g., at least about 20 or 30 contiguous nucleotides, more usually about 40 contiguous nucleotides, and preferably more than about 50 contiguous nucleotides. Selective hybridization exists when hybridization occurs which is more selective than total lack of specificity. See, Kanehisa, *Nucleic Acid Res.* 12:203-213 (1984).

Nucleic acids of the present invention include RNA, cDNA, genomic DNA, synthetic forms and mixed polymers, both sense and antisense strands. Furthermore, different alleles of each isoform are also included. The present invention also provides recombinant nucleic acids which are not otherwise naturally occurring. The nucleic acids included in the present invention will typically comprise RNA or DNA or mixed polymers. The DNA compositions will generally include a coding region which encodes a polypeptide comprising an amino acid sequence substantially homologous to the amino acid sequence of a PfEMP1 protein. More preferred are those DNA segments comprising a nucleotide sequence which encodes a CD36 binding fragment of the PfEMP1 protein.

cDNA encoding the polypeptides of the present invention, or fragments thereof, may be readily employed as a probe useful for obtaining genes which encode the PfEMP1 polypeptides of the present invention. Preparation of these

probes may be carried out by generally well known methods. For example, the cDNA probes may be prepared from the amino acid sequence of the PfEMP1 protein. In particular, probes may be prepared based upon segments of the amino acid sequence which possess relatively low levels of degeneracy, i.e., few or one possible nucleic acid sequences which encode therefor. Suitable synthetic DNA fragments may then be prepared, e.g., by the phosphoramidite method described by Beaucage and Carruthers, *Tetra. Letts.* 22:1859-1862 (1981). Alternatively, nucleotide sequences which are relatively conserved among the PfEMP1 coding sequences for the various *P. falciparum* strains may be used as suitable probes. A double stranded probe may then be obtained by either synthesizing the complementary strand and hybridizing the strands together under appropriate conditions or by adding the complementary strand using DNA polymerase with an appropriate primer sequence. Such cDNA probes may be used in the design of oligonucleotide probes and primers for screening and cloning such genes, e.g., using well known PCR techniques, or, alternatively, may be used to detect the presence or absence of a PfEMP1 gene in a cell. Such nucleic acids, or fragments may comprise part or all of the cDNA sequence that encodes the polypeptides of the present invention. Effective cDNA probes may comprise as few as 15 consecutive nucleotides in the cDNA sequence, but will often comprise longer segments. Further, these probes may further comprise an additional nucleotide sequence, such as a transcriptional primer sequence for cloning, or a detectable group for easy identification and location of complementary sequences.

cDNA or genomic libraries of various types may be screened for new alleles or related sequences using the above probes. The choice of cDNA libraries normally corresponds to tissue sources which are abundant in mRNA for the desired polypeptides. Phage libraries are normally preferred, e.g.,  $\lambda$ gt11, but plasmid or YAC libraries may also be used. Clones of a library are spread onto plates, transferred to a substrate for screening, denatured, and probed for the presence of the desired sequences.

In a related aspect, the nucleic acids of the present invention also include the PCR product or RT-PCR product, produced using the above described primer probes. For example, primer probes derived from the nucleotide sequence shown in Figure 12, may be used to amplify sequences from different malaria parasites, and in particular, different strains of *P. falciparum*. Examples of particularly preferred nucleic acid sequences include those nucleic acid sequences which are PCR amplified using the following oligonucleotide probes:

```

5'      1a:  AAGGAAGACAAAATTATGTCCTAT
          25:  AATGGAGAGACGAACATGG
          53:  TCCAAAAATGGGTTGAACAAAA
          80:  ATTGGACTCATGATGATTTTC
          96:  TTTTGGAAATTATTCAGGATACT
          135: CTAAAGGTTTTGTGCTGCTGAAA
          156: AAGCCGATAAATGCCTAAAAAC

3'      59:  TTTTTGTTCAACCCATTTTTGGA
          87:  TGAAGAAAATCATCATGAGTCCA
          102: AGTATCCTGAATAATTTCCAA
          140: TTCAGCGACAAAACCTTTAGT
          179: GAGCGGGCGACACTTCTATCT
          192: CTTAGGGTCGGCAGGTGGTG
          233: ATCCGTCTTTTCCTCCTGGACTT

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- a. The number designation indicates the amino acid position within amino acids 575 through 808 of the amino acid sequence shown in Figure 2 and 12, which is encoded by the respective end of the probe (3' or 5').

Included among the most preferred of the above described nucleic acid sequences are the nucleic acids which are PCR amplified using the following primer probe combinations: 5'-1: 3'59, 3'140, 3'179; 5'53: 3'140, 3'179; and 5'140:3'179.

Also included among the most preferred oligonucleotides are those nucleic acid segments which encode the relatively conserved peptides described above. Examples of these oligonucleotides which have been identified from the previously described *P. falciparum* strains are shown in Table 1, below:

Table 1

<i>Plasmodium falciparum</i> Strain	3' primer									
MC type	ACT	ACA	ATT	GAT	AAA	TTA	CTA	CAA	CAC	GAA
ITG type	ACC	ACA	ATT	GAT	AAA	TTG	CTC	AAT	CAC	GAA
HB-3 type	ACT	ACA	ATT	GAT	AAA	ATA	CTA	CAA	CAC	GAA
Degenerate Universal Primers	ACC	ACA	ATT	GAT	AAA	TTA	CTA	CAA	CAC	GAA
	T					A G	C A T			
	5' Primer									
MC type	TTT	TTT	TGG	ATG	TGG	GTA	CAT	GAT	ATG	TTA
ITG type	TTT	TTT	TGG	AAG	TGG	GTT	ACC	GAA	ATG	TTA
HB-3 type	TTT	TTT	TGG	GAT	TGG	GTT	TAT	CAT	ATG	TTA
Degenerate Universal Primers	TTT	TTT	TGG	GAG	TGG	GTA	TAT	GAT	ATG	TTA
				ATT			T	ACC	C A	
							C			

In the synthesis of the universal primer sequences, single sequence lines indicate the primary sequence of the primer. Where two bases are shown for a single position, e.g., A and T, it refers to a step in the synthesis of the primer sequence where equal amounts of each base were added to the synthesis step, resulting in equal amounts of each base being coupled to growing oligomers in that position. Similarly, where three bases are shown for a given position, equal amounts of the three bases are added to the synthesis step. This results in a mixture of oligonucleotide sequences having all possible combinations of sequences reflecting the multiple bases at each of the indicated positions. In some cases, expression of the full length primer required the

addition of additional bases to the 5' primer, e.g., a CTT before the TTT, to correct for truncation problems upon inserting the primer into the vector used.

Thus, based upon the above sequences, the general structure of the universal 3' primer sequence can be described as a mixture of a number of individual primer sequences where each individual primer has the following general structure:

ACX<sub>6</sub>ACA ATT GAT AAA X<sub>7</sub>TX<sub>8</sub> CTX<sub>9</sub> X<sub>10</sub>AX<sub>11</sub> CAC GAA

where X<sub>6</sub> is selected from C and T, X<sub>7</sub> is selected from T and A, X<sub>8</sub> is selected from G and A, X<sub>9</sub> is selected from C and A, X<sub>10</sub> is selected from C and A and X<sub>11</sub> is selected from T and A.

Similarly, each of the individual primer sequences within the universal 5' primer is represented by the general structure:

TTT TTT TGG X<sub>12</sub>X<sub>13</sub>X<sub>14</sub> TGG GTX<sub>15</sub> X<sub>16</sub>X<sub>17</sub>X<sub>18</sub> X<sub>19</sub>AX<sub>20</sub> ATG TTA

where X<sub>12</sub> is selected from G and A, X<sub>13</sub> is selected from A and T, X<sub>14</sub> is selected from G and T, X<sub>15</sub> is selected from A and T, X<sub>16</sub> is selected from of T, A and C, X<sub>17</sub> is selected from A and C, X<sub>18</sub> is selected from of T and C, X<sub>19</sub> is selected from G and C and X<sub>20</sub> is selected from T and A.

The above-described universal primer sequences are particularly useful in identifying corresponding gene sequences in different strains of *P. falciparum*, as well as in the design of particularly preferred peptides of the invention.

The above universal primers may be particularly useful in generating a "finger print" identification of individual *P. falciparum* cells and clones by amplifying a distinct set of PCR products of varying sizes from the var genes and/or the expressed var genes of these cells and clones.

The nucleic acids of the present invention may be present in whole cells, cell lysates or in partially pure or substantially pure or isolated form. Such "substantially



pur " or "isolated" forms of these nucleic acids generally refer to the nucleic acid separated from contaminants with which it is generally associated, e.g., lipids, proteins and other nucleic acids. The nucleic acids of the present invention will be greater than about 50% pure. Typically, the nucleic acids will be more than about 60% pure, more typically, from about 75% to about 90% pure, and preferably, from about 95% to about 98% pure.

The present invention also provides substantially similar nucleic acid sequences, allelic variations and natural or induced sequences of the above described nucleic acids, as well as chemically modified and substituted nucleic acids, e.g., those which incorporate modified nucleotide bases or which incorporate a labelling group.

In addition to comprising a segment which encodes a PfEMP1 protein or fragment thereof, the nucleic acids of the present invention may also comprise a segment encoding a heterologous protein, such that the gene is expressed to produce the two proteins as a fusion protein, as substantially described above.

In addition to their use as probes, the nucleic acids of the present invention may also be used in the preparation of the polypeptides of the present invention, as described above. DNA encoding the polypeptides of the present invention will typically be incorporated into DNA constructs capable of introduction to and expression in an *in vitro* cell culture. Often, the nucleic acids of the present invention may be used to produce a suitable recombinant host cell. Specifically, DNA constructs will be suitable for replication in a unicellular host, such as bacteria, e.g., *E. coli*, viruses or yeast, but may also be intended for introduction into a cultured mammalian, plant, insect, or other eukaryotic cell lines. DNA constructs prepared for introduction into bacteria or yeast will typically include a replication system recognized by the host, the intended DNA segment encoding the desired polypeptide, transcriptional and translational initiation and termination regulatory sequences operably linked to the polypeptide encoding segment. A DNA segment is

operably linked when it is placed into a functional relationship with another DNA segment. For example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence; DNA for a signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide. Generally, DNA sequences that are operably linked are contiguous, and in the case of a signal sequence both contiguous and in reading phase. However, enhancers need not be contiguous with the coding sequences whose transcription they control. Linking is accomplished by ligation at convenient restriction sites or at adapters or linkers inserted in lieu thereof. The selection of an appropriate promoter sequence will generally depend upon the host cell selected for the expression of the DNA segment. Examples of suitable promoter sequences include prokaryotic, and eukaryotic promoters well known in the art. See, e.g., Sambrook et al., *supra*. The transcriptional regulatory sequences will typically include a heterologous enhancer or promoter which is recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters are known and available. See Sambrook et al., *supra*.

Conveniently available expression vectors which include the replication system and transcriptional and translational regulatory sequences together with the insertion site for the PfEMP1 polypeptide encoding segment may be employed. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al., *supra*, and in Metzger et al., *Nature* 334:31-36 (1988).

The vectors containing the DNA segments of interest, e.g., those encoding polypeptides comprising a PfEMP1 protein or fragments thereof, can be transferred into the host cell by well known methods, which may vary depending upon the type of host used. For example, calcium chloride transfection is commonly used for prokaryotic cells, whereas calcium phosphate treatment may be used for other hosts. See, Sambrook et al.,

*supra*. The term "transformed cell" as used herein, includes the progeny of originally transformed cells.

Techniques for manipulation of nucleic acids which encode the polypeptides of the present invention, *i.e.*, subcloning the nucleic acids into expression vectors, labeling probes, DNA hybridization and the like, are generally described in Sambrook, et al., *supra*.

In recombinant methods, generally the nucleic acid encoding a peptide of the present invention is first cloned or isolated in a form suitable for ligation into an expression vector. After ligation, the vectors containing the nucleic acids fragments or inserts are introduced into a suitable host cell, for the expression of the polypeptide of the invention. The polypeptides may then be purified or isolated from the host cells. Methods for the synthetic preparation of oligonucleotides are generally described in Gait, *Oligonucleotide Synthesis: A Practical Approach*, IRL Press (1990).

There are various methods of isolating the nucleic acids which encode the polypeptides of the present invention. Typically, the DNA is isolated from a genomic or cDNA library using labeled oligonucleotide probes specific for sequences in the desired DNA. Restriction endonuclease digestion of genomic DNA or cDNA containing the appropriate genes can be used to isolate the DNA encoding the binding domains of these proteins. From the PfEMP1 sequence given in Figure 12, a panel of restriction endonucleases can be constructed to give cleavage of the DNA in desired regions, *i.e.*, to obtain segments which encode biologically active fragments of the PfEMP1 protein. Following restriction endonuclease digestion, DNA encoding the polypeptides of the present invention is identified by its ability to hybridize with a nucleic acid probe in, for example a Southern blot format. These regions are then isolated using standard methods. See, *e.g.*, Sambrook, et al., *supra*.

The polymerase chain reaction, or "PCR" can also be used to prepare nucleic acids which encode the polypeptides of the present invention. PCR technology is used to amplify

nucleic acid sequences of the desired nucleic acid, e.g., the DNA which encodes the polypeptides of the invention, directly from mRNA, cDNA, or genomic or cDNA libraries.

5       Appropriate primers and probes for amplifying the  
nucleic acids described herein, may be generated from analysis  
of the PfEMP1 oligonucleotide sequence, such as those shown in  
Figure 12 and Table 1. Briefly, oligonucleotide primers  
complementary to the two 3' borders of the DNA region to be  
10       amplified are synthesized. The PCR is then carried out using  
the two primers. See, e.g., *PCR Protocols: A Guide to Methods  
and Applications* (Innis, M., Gelfand, D., Sninsky, J. and  
White, T., eds.) Academic Press (1990). Primers can be  
selected to amplify various sized segments from the PfEMP1  
oligonucleotide sequence. The primers may also contain a  
15       restriction site and additional bases to permit "in-frame"  
cloning of the insert into an appropriate expression vector,  
using the restriction sites present on the primers.

### III. Antibodies

20       The nucleic acids and polypeptides of the present  
invention, or fragments thereof, are also useful in producing  
antibodies, either polyclonal or monoclonal. These antibodies  
are produced by immunizing an appropriate vertebrate host,  
e.g., rat, mouse, rabbit or goat, with a polypeptide of the  
25       invention, or its fragment, or plasmid DNA containing a  
nucleic acid of the invention, alone or in conjunction with an  
adjunct. Usually, two or more immunizations are involved, and  
a few days following the last injection, the blood or spleen  
of the host will be harvested.

30       For production of polyclonal antibodies, an  
appropriate target immune system is selected, typically a  
mouse or rabbit, but also including goats, sheep, cows, guinea  
pigs, monkeys and rats. The substantially purified antigen or  
plasmid is presented to the immune system in a fashion  
35       determined by methods appropriate for the animal. These and  
other parameters are well known to immunologists. Typically,  
injections are given in the footpads, intramuscularly,  
intradermally or intraperitoneally. Th immunoglobulins

produced by the host can be precipitated, isolated and purified by routine methods, including affinity purification.

For monoclonal antibodies, appropriate animals will be selected and the desired immunization protocol followed.

5 After the appropriate period of time, the spleens of these animals are excised and individual spleen cells are fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter, the cells are clonally separated and the supernatants of each clone are tested for  
10 the production of an appropriate antibody specific for the desired region of the antigen. Techniques for producing antibodies are well known in the art. See, e.g., Goding et al., *Monoclonal Antibodies: Principles and Practice* (2d ed.) Acad. Press, N.Y., and Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York  
15 (1988). Other suitable techniques involve the *in vitro* exposure of lymphocytes to the antigenic polypeptides or alternatively, to selection of libraries of antibodies in phage or similar vectors. Huse et al., *Generation of Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda*, *Science* 246:1275-1281 (1989). Monoclonal  
20 antibodies with affinities of  $10^8$  liters/mole, preferably  $10^9$  to  $10^{10}$  or stronger, will be produced by these methods.

The antibodies generated can be used for a number of  
25 purposes, e.g., as probes in immunoassays, for inhibiting PfEMP1 binding to its ligands, thereby inhibiting or reducing erythrocyte sequestration, in diagnostics or therapeutics, or in research to further elucidate the mechanism of various aspects of malarial infection, and particularly, *P. falciparum*  
30 infection.

The antibodies of the present invention can be used with or without modification. Frequently, the antibodies will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. Such labels  
35 include those that are well known in the art, such as the labels described previously for the polypeptides of the invention. Additionally, the antibodies of the invention may be chimeric, human-like or humanized, in order to reduce their

potential antigenicity, without reducing their affinity for their target. Chimeric, human-like and humanized antibodies have generally been described in the art. Generally, such chimeric, human-like or humanized antibodies comprise variable regions, e.g., complementarity determining regions (CDR) (for humanized antibodies), from a mammalian animal, i.e., a mouse, and a human framework region. By incorporating as little foreign sequence as possible in the hybrid antibody, the antigenicity is reduced. Preparation of these hybrid antibodies may be carried out by methods well known in the art.

Preferred antibodies are those that are specifically immunoreactive with the polypeptides of the present invention and their immunologically active fragments. The phrase "specifically immunoreactive," when referring to the interaction between an antibody of the invention and a particular protein, refers to an antibody that specifically recognizes and binds with relatively high affinity to the particular protein, such that this binding is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

The antibodies generated can be used for a number of purposes, e.g., as probes in immunoassays, for inhibiting interaction between a PfEMP1 protein and its ligand, e.g., CD-36, TSP, ICAM-1, VCAM-1, ELAM-1, or Chondroitin sulfate, thereby inhibiting or reducing the level of PfEMP1-ligand interaction, in diagnostics or therapeutics, or in research to

further elucidate the mechanism of malarial pathology, e.g., erythrocyte sequestration. Where the antibodies are used to block or reverse the interaction between a polypeptide of the invention and an associating ligand or PE, the antibody will generally be referred to as a "blocking antibody."

Preferred antibodies are those monoclonal or polyclonal antibodies which specifically recognize and bind the polypeptides of the invention. Accordingly, these preferred antibodies will specifically recognize and bind the polypeptides which have an amino acid sequence that is substantially homologous to the amino acid sequence shown in Figures 2, 20 or 21, or immunologically active fragments thereof. Still more preferred are antibodies which are capable of forming an antibody-ligand complex with the relatively conserved polypeptide fragments of PfEMP1 sequences, and are thereby capable of blocking an interaction of PfEMP1 from a variety of *P. falciparum* strains, and PfEMP1 ligands.

#### IV. Methods of Use

The polypeptides, antibodies, and nucleic acids of the present invention have a variety of important uses, including, but not limited to, diagnostic, screening, prophylactic, including vaccination, and therapeutic applications.

##### A. Diagnostic Applications

In a particularly preferred aspect, the present invention provides methods and reagents useful in detecting the presence of PfEMP1 in a sample. These detection methods are particularly useful in diagnosing malarial infections in a patient.

For example, in a particularly preferred aspect, the antibodies of the present invention may be used to assay for the presence or absence of PfEMP1 in a sample. Immunoassay techniques for the detection of the particular antigen are very well known in the art. For a review of immunological and immunoassay procedures in general, see *Basic and Clinical Immunology* 7th Edition (D. Stites and A. T r r ed.) 1991.

Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in *Enzyme Immunoassay*, E.T. Maggio, ed., CRC Press, Boca Raton, Florida (1980); "Practice and Theory of Enzyme Immunoassays," P. Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V. Amsterdam (1985); and, Harlow and Lane, *Antibodies, A Laboratory Manual*, supra. Generally, these methods comprise contacting the antibody with a sample to be tested, and detecting any specific binding between the antibody and a protein within the sample. Typically, this will be in a blot format, e.g., western blot, or in an ELISA format. Methods of performing these assay formats are well known in the art. See, e.g., *Basic and Clinical Immunology*, 7th ed. (D. Stites and A. Terr, eds., 1991).

Typically, these diagnostic methods comprise contacting a sample with an antibody to PfEMP1, as described herein, and determining whether the antibody binds to any portion of the sample. In the case of human diagnostic techniques, the sample may be a whole blood sample, or some fraction thereof, e.g. an erythrocyte containing sample. Generally, such diagnostic methods are well known in the art, and are described in the above described references. The immunoreactivity of the antibody with the sample, indicates the presence of PfEMP1 in the sample, and, in the case of a sample derived from a patient, a possible malarial infection.

Alternatively, labeled polypeptides of the present invention may be used as diagnostic reagents in detecting the presence or absence of antibodies to PfEMP1, in a patient. The presence of antibodies within a patient would be indicative that the patient had been exposed to a malaria parasite sufficiently to result in an antigenic response.

Similarly, the nucleic acid probes of the invention may be used in a similar manner, i.e., to identify the presence in a sample of a DNA segment encoding a PfEMP1 polypeptide, or as PCR or RT-PCR primers to amplify and then detect PfEMP1 encoding nucleic acid segments. Such assays typically involve the immobilization of nucleic acids in the



sample, followed by interrogation of the immobilized sequences with a chemically labeled oligonucleotide probe, as described herein. Hybridization of the probe to the immobilized sample indicates the presence of a DNA segment encoding PfEMP1, and thus, a malarial infection. As described above, assays may be further designed to indicate not only the presence of a Malarial parasite, but also indicate the strain of parasite present. Although described in terms of an immobilized sample probed with a solution based oligonucleotide probe, a wide variety of assay conformations may be adopted, which conformations are generally well known in the art.

#### B. Screening Applications

In another particularly preferred aspect, the present invention provides methods for screening compounds to determine whether or not the particular compound is an antagonist of a symptom of a malarial infection. In particular, the screening methods of the present invention can be used to determine whether a test compound is an antagonist of the sequestration of erythrocytes which is associated with *P. falciparum* malaria. More particularly, the screening methods can determine whether a compound is an antagonist of the PfEMP1/ligand interaction. Ligands of PfEMP1 generally include, e.g., CD36, TSP, ELAM-1, ICAM-1, VCAM-1 or Chondroitin sulfate.

Generally, the screening methods of the present invention comprise contacting PfEMP1 protein, or a fragment thereof, and/or ligand protein, with a compound which is to be screened ("test compound"). The level of PfEMP1/ligand complex formed may then be detected and compared to a control, e.g., in the absence of the test compound. A decrease in the level of PfEMP1/ligand interaction is indicative that the test compound is an antagonist of that interaction.

A test compound may be a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials, such as bacteria, phage, yeast, plants, fungi, animal cells or tissues. Test compounds are evaluated for potential activity as antagonists

of PfEMP1/ligand interaction by inclusion in the screening assays described herein. An "antagonist" refers to a compound which will diminish the level of PfEMP1/ligand interaction, over a control.

5           It will often be desirable in the screening assays of the present invention, to provide one of the PfEMP1 or ligand proteins immobilized on a solid support. Suitable solid supports include, e.g., agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose, polystyrene, 10 filter paper, nitrocellulose, ion exchange resins, plastic films, glass beads, polyaminemethylvinylether maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The support may be in the form of, e.g., a test tube, microtiter plate, beads, test strips, 15 flat surface, e.g., for blotting formats, or the like. The reaction of the PfEMP1 polypeptide or its ligand with the particular solid support may be carried out by methods well known in the art, e.g., binding to an immobilized anti-PfEMP1 antibody, or binding to prederivatized solid support.

20           In addition to the foregoing, it may also be desirable to provide either the PfEMP1 or its ligand linked to a suitable detectable group to make detection of binding of one protein to the other, simpler. Useful detectable groups, or labels, are generally well known in the art. For example, 25 a detectable group may be a radiolabel, such as,  $^{125}\text{I}$ ,  $^{32}\text{P}$  or  $^{35}\text{S}$ , or a fluorescent or chemiluminescent group. Alternatively, the detectable group may be a substrate, cofactor, inhibitor, affinity ligand, antibody binding epitope tag, or an enzyme which is capable of being assayed. Suitable 30 enzymes include, e.g., horseradish peroxidase, luciferase, or another readily assayable enzymes. These enzyme groups may be attached to the PfEMP1 polypeptide, or its ligand by chemical means or maybe expressed as a fusion protein, as already described.

35           Generally, where one of the above proteins, e.g., the PfEMP1 ligand, is immobilized on a solid support, the other protein, e.g., PfEMP1 or its fragment, will be labelled with an appropriate detectable group. Assaying whether a

compound is an antagonist of the interaction of the two proteins is then a matter of contacting the labelled PfEMP1 polypeptide or fragment with the immobilized ligand, in the presence of the test compound, under conditions which allow specific binding of the two proteins. The amount of label bound to the solid support is compared to a control, where no test compound was added. Where a test compound results in a reduction of the amount of label which binds to a solid support, that compound is an antagonist of the PfEMP1/ligand interaction.

C. Therapeutic and Prophylactic Applications

In addition to the above described uses, the polypeptides of the present invention may also be used in therapeutic applications, for the treatment of human and/or non-human mammalian patients. The therapeutic uses of the polypeptides of the present invention include the treatment of symptoms of existing disorders, as well as prophylactic applications. The term "prophylactic" refers to the prevention of a particular disorder, or symptoms of a particular disorder. Thus, prophylactic treatments will generally include drugs which actively participate in the prevention of a particular disorder such as a malaria infection, or symptoms thereof. Prophylactic applications will also include treatments which elicit a preventative response from a patient, including, for example, an immunological response as in the case of vaccination.

Typically, both therapeutic and prophylactic applications will comprise administering an effective amount of the compositions of the present invention to a patient, to treat or prevent symptoms, or the onset of a malarial parasite infection. An "effective amount", as the term is used herein, is defined as the amount of the composition which is necessary to achieve the desired goal, i.e. alleviation of symptoms, prevention of symptoms or infection, or treatment of disease.

In prophylactic applications, the polypeptides of the present invention may be used in a variety of treatments. For example, the polypeptides of the invention are particularly useful as a vaccine, to elicit an immunological

respons by a patient, e.g., production of antibodies sp cific for PfEMP1. In particular, such vaccine applications generally involve the administration of the PfEMP1 protein or biologically active fragments thereof, to the host or patient.

5 In response to this administration, the patient's immune system will generate antibodies to the particular PfEMP1 protein or fragment introduced. An amount of the polypeptides sufficient to produce an immunological response in a patient is termed "an immunogenically effective amount." Thus, the

10 vaccines of the present invention will contain an immunogenically effective amount of the polypeptides of the present invention. The immune response of the patient may include generation of antibodies, activation of cytotoxic T-lymphocytes against cells expressing the polypeptides, e.g.,

15 PE, or other mechanisms known to the skilled artisan. See, e.g., Paul, *Fundamental Immunology*, 2d Edition, Raven Press. Useful carriers are well known in the art, and include for example, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(D-lysine; D-

20 glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine. The vaccines can also contain a physiologically tolerable diluent, such as water, buffered water, buffered saline, saline and typically may further include an adjuvant, such as incomplete Freunds

25 adjuvant, aluminum phosphate, aluminum hydroxide, alum, or other materials well known in the art.

Alternatively, the nucleic acids of the present invention may also be used as vaccines for the prevention of malaria symptoms, and/or infection by malaria parasites. See

30 Sedegah, et al. *Proc. Nat'l Acad. Sci.* (1994) 91:9866-9870. For example, plasmid DNA comprising the nucleic acids of the present invention may be directly administered to a patient. Expression of this "naked" DNA will have effects similar to the injection of the actual polypeptides, as described above.

35 Specifically, the patient's immune response to the presence of the proteins express d from the DNA, will result in the production of antibodies to that protein. The nucleic acids may also be used to design antisense probes to interupt

transcription of PfEMP1 peptides in parasitized erythrocytes. Antisense methods are generally well known in the art.

The polypeptides of the present invention, and analogs thereof, may also be used as prophylactic treatments to prevent the onset of symptoms of malarial infection. For example, administration of the polypeptides can directly inhibit, block or reverse the sequestration of erythrocytes in patients suffering from *P. falciparum* malaria infections. In particular, the polypeptides of the invention may be used to compete with or displace PE associated PfEMP1 in binding CD36. The blockage or reversal of sequestration will reduce or eliminate the microvascular occlusion generally associated with the pathology of this type of malaria, which, again, can lead to destruction of the PE by the host.

The antibodies of the invention may also be used in a similar fashion. In particular, the antibodies, which are capable of binding the polypeptides of the present invention, may be directly administered to a patient. By binding PfEMP1, the antibodies of the present invention are effective in blocking, reducing or reversing PfEMP1 mediated interactions, e.g., erythrocyte sequestration. Chimeric, human-like or humanized antibodies are particularly useful for administration to human patients. Additionally, such antibodies may also be used as a passive vaccination method to provide a subject with a short term immunization, much as anti-hepatitis A injections have been used previously.

In alternative aspects, the polypeptides, antibodies and nucleic acids of the invention may be used to treat a patient already suffering from a malarial infection. In particular, the compositions of the present invention may be administered to a patient suffering from a malarial infection to treat symptoms associated with that infection. More particularly, these compositions may be administered to the patient to prevent or reduce erythrocyte sequestration and the resulting microvascular occlusion associated with malarial, and more specifically, *P. falciparum*, infections.

Although the polypeptides, nucleic acids and antibodies of the present invention may be administered alone,

for therapeutic and prophylactic applications, these elements will generally be administered as part of a pharmaceutical composition, e.g., in combination with a pharmaceutically acceptable carrier. Typically, a single composition may be  
5 used in both therapeutic and prophylactic applications. Pharmaceutical formulations suitable for use in the present invention are generally described in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., 17th ed. (1985).

The pharmaceutical compositions of the present  
10 invention are intended for parenteral, topical, oral, or local administration. Where the pharmaceutical compositions are administered parenterally, the invention provides pharmaceutical compositions that comprise a solution of the agents described above, e.g., polypeptides of the invention,  
15 dissolved or suspended in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, saline glycine, and the like. These compositions may be sterilized by conventional, well known methods, e.g., sterile filtration.  
20 The resulting aqueous solutions may be packaged for use as is, or lyophilized for combination with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting  
25 and buffering agents, tonicity adjusting agents, wetting agents, and the like, for example sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid  
30 carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic  
35 composition may be formed by incorporating any of the normally employed excipients, such as the previously listed carriers, and generally, 10-95% of active ingredient, and more preferably 25-75% active ingredient. In addition, for oral

administration of peptide based compounds, the pharmaceutical compositions may include the active ingredient as part of a matrix to prevent proteolytic degradation of the active ingredient by digestive process, e.g., by providing the pharmaceutical composition within a liposomal composition, according to methods well known in the art. See, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., 17th Ed. (1985).

For aerosol administration, the polypeptides are generally supplied in finely divided form along with a surfactant or propellant. Preferably, the surfactant will be soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids, with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

The above described compositions are suitable for a single administration or a series of administrations. When given as a series, e.g., as a vaccine booster, the inoculations subsequent to the initial administration are given to boost the immune response, and are typically referred to as booster inoculations.

The amount of the above compositions to be administered to the patient will vary depending upon what is to be administered to the patient, the state of the patient, the manner of administration, and the particular application, e.g., therapeutic or prophylactic. In therapeutic applications, the compositions are administered to the patient already suffering from a malarial infection, in an amount sufficient to inhibit the spread of the parasite through the erythrocytes, and thereby cure or at least partially arrest the symptoms of the disease and its associated complications. An amount adequate to accomplish this is termed "a therapeutically effective amount." Amounts effective for this use will depend upon the severity of the disease and the

weight and general state of the patient, but will generally be in the range of from about 1 mg to about 5 g of active agent per day, preferably from about 50 mg per day to about 500 mg per day, and more preferably, from about 50 mg to about 100 mg per day, for a 70 kg patient.

For prophylactic applications, immunogenically effective amounts will also depend upon the composition, the manner of administration and the weight and general state of the patient, as well as the judgment of the prescribing physician. For the peptide, peptide analog and antibody based pharmaceutical compositions, the general range for the initial immunization (for either prophylactic or therapeutic applications) will be from about 100  $\mu$ g to about 1 g of polypeptide for a 70 kg patient, followed by boosting doses of from about 1  $\mu$ g to about 1 gm of polypeptide pursuant to a boosting regimen over weeks to months, depending upon the patient's response and condition, e.g., by measuring the level of parasite or antibodies in the patient's blood. For nucleic acids, typically from about 30 to about 100  $\mu$ g of nucleic acid is injected into a 70 kg patient, more typically, about 50 to 150  $\mu$ g of nucleic acid is injected, followed by boosting treatments as appropriate.

The present invention is further illustrated by the following examples. These examples are merely to illustrate aspects of the present invention and are not intended as limitations of this invention.

## V. EXAMPLES

### Example 1- General Procedures

#### A. Parasites

The Malayan Camp MC K+R+C+ (knob, rosetting and cytoadherence positive) line of *P. falciparum*, denoted MC K+, the ItG2-ICAM K+C+ clone which binds to CD36, TSP, and ICAM-1 (Ockenhouse et al., *J. Infect. Dis.* (1991) 164:163-169), and the ItG2-F6 (K+ C+ [CD36, TSP, ICAM-1]) were maintained in culture with O+ RBC (Handunnetti et al., *Am J. Trop. Med. Hyg.* (1992) 46:371-381). DNA or proteins were also extracted from the following *P. falciparum* parasites in human erythrocytes:



MC K+C+R-; MC K-C-; ItG<sub>2</sub>-G1 (K+C+); FCR<sub>3</sub>/C5 (K+C+); FCR<sub>3</sub>/C6 (K-C-); 7G8 (K+); D10(K+C+ [TSP only]); Palo Alto, PA (K-C+); HB3 (K+C+ [TSP only]) and Dd2 (K+, low C+). FVO(K+C+) was derived from Aotus monkey 1150. Unless indicated, C+ refers to adherence to both CD36 and TSP.

B. Preparation of Nucleic Acids

*P. falciparum* gDNA was isolated from mature PE by lysis in NP40 (Pasloske et al., *Molec. Biochem. Parasitol.* (1993) 59:59-72). DNA for Southern blotting was digested with Eco RI or Eco RI/Hind III and blotted under high stringency (Pasloske, et al., *supra*).

C. Screening OF MC gDNA Expression Library

The genomic DNA  $\lambda$ gt11 expression library and screening of the library with antibodies has been described (Pasloske et al., *supra*). Rabbit serum 05-75 (van Schravendijk et al., *Am. J. Trop. Med. Hyg.* (1993) 49:552-565) was exhaustively adsorbed with the  $\lambda$ gt11-12.1.3 clone (Pasloske et al.) and used at 1:150 dilution for screening the gDNA library. Clones so derived were subcloned into the SK-vector (Stratagene, Cloning Systems, La Jolla, CA).

D. Isolation Of cDNA Clones

The MC K+ cDNA library was prepared in the expression plasmid pJFE14DAF (Alamo et al., manuscript submitted) as described by (Elliott et al., *Proc. Nat'l Acad. Sci.* (1990) 87:6363-6367). PfEMP1 clones were isolated from the cDNA library using a modified version of the "leapfrog" method (Gibbons et al., *Proc. Nat'l Acad. Sci.* (1991) 88:8563-8567). cDNA clones extending 5' or 3' from previously isolated DNA clones were generated by PCR using an oligonucleotide primer 100-150 bases from the proximal terminus region of the cloned sequence and another primer derived from the vector arm flanking the cloning site. PCR was performed on 10 ng of the cDNA library using 30 cycles with a profile of 1 min. at 94°C, 1 min. at 55°C and 2.5 min. at 72°C. The PCR products were fractionated on low melt agarose gels and products of 1-3 kb were gel purified (Wizard PCR preps, Promega, Madison, WI), cloned into pAMP vector (Life Technologies Inc. Gaithersburg, MD) and transformed into

*E. coli* DH5- $\alpha$  Max. Efficiency cells (Life Technologies, Gaithersburg, MD) or Sure-2 cells (Stratagene cloning Systems, La Jolla, CA). To facilitate cloning, the PCR primers were designed with 5' adaptor sequences for directional insertion into the pAMP vector. PfEMP1 clones were identified by direct colony hybridization using end-labeled oligonucleotides designed 50-100 bases internal to the sequence specific PCR primer.

#### E. DNA Sequencing and Analysis

Plasmid DNA was isolated from the recombinant clones using the Wizard Miniprep DNA isolation system (Promega, Madison, WI), alkali denatured and sequenced via the dideoxy chain termination method using the Sequenase kit (USB, Cleveland, Ohio). Either vector specific or custom oligonucleotides primers were used for primer directed sequencing. For some of the clones, the insert was subcloned into the pBluescript SK-vector (Stratagene cloning system, La Jolla, CA) and unidirectional deletion mutants generated using Exonuclease III (Henikoff, Gene (1984) 28:351-359). To ensure that new clones were continuous with the initial ones, primers from within the two clones were used to generate PCR products from MC gDNA and the cDNA library and the identity of the generated product was confirmed by size, hybridization with oligonucleotides and direct sequencing (fmol sequencing system, Promega, Madison, WI). Sequences were analyzed using DNASTAR (DNASTAR Inc., Madison, WI) sequence analysis software.

#### F. Recombinant Fusion Proteins

GST fusion proteins and Maltose Binding Protein (MBP) fusion proteins were constructed by cloning of PCR products carrying a Bam HI site at the 5' end and an EcoRI site at the 3' end of the BamHI/EcoRI sites of the pGEX-3X vector (Pharmacia Biotech) for GST and into modified pMAL vector (New England Biolab) for MBP fusion proteins. The recombinant fusion proteins generated were: rA1 (a.a.3-158), rB1 (a.a. 161-385), rC1-1 (a.a. 402-605), rC1-2 (a.a. 576-808), rD1 (a.a. 818-1003), rD2 (982-1320), rF1-1 (1300-1707), rF1-2 (1688-2190), rF1-3 (2171-2450), rG1 (2550-2794), rD3

(992-1243), rE1-1 (1219-1471), rE1-2 (1454-1719), and rGST (See Figure 15A). Clone F1-2 was cloned into SmaI/NotI sites of the pMal vector and expressed as an MBP fusion only. The fusion proteins were expressed in *E. coli* DH5- $\alpha$  or Sure-2 cells. The GST fusion proteins were purified on glutathione-Sepharose (Pharmacia, LKB Biotechnology, Piscataway, NJ) (van Schravendijk et al., *supra*) except that PBS was replaced with a column buffer (20 mM Tris, 200 mM NaCl, pH 7.5). MBP fusion proteins were purified on Amylose Resin (New England Biolab) according to the manufacturer's procedure. rA62-5 was derived from clone A62 and rPfEMP3 corresponding to the 12.1.3 RP (van Schravendijk et al.).

#### G. Antibodies

Mouse MAb 179 recognizes an epitope sequence incorporated into the carboxy terminus of SCD36 expressed as phosphoinositol glycan-linked extracellular domain (Affymax Research Institute). Mouse MAb 141 recognizes GST. Adherence blocking anti-CD36 MAb 8A6 (Barnwell et al., *J. Clin. Invest.* (1989) 84:765-772) was a gift from Dr. J. Barnwell (New York Medical Center, NY). Rabbit serum 05-75 which recognizes both PfEMP3 and MC PfEMP1 was described previously (Schravendijk et al., *supra*). A human immune serum pool was prepared from five individuals resident in a *P. falciparum* endemic area of Ghana (Schravendijk et al., *supra*). Aotus anti-*P. falciparum* sera 779 and 9050 were derived from animals infected with the Aotus MC K+ strain and drug cured (Leech et al., *J. Exp. Med.* (1984) 159:1567-1575).

RP (as listed above, except for F1-2) in solution or bound to the purification resin, e.g., glutathione-Sepharose 4B beads (Pharmacia) or Amylose Resin, were used to immunize goats (1 mg RP), rabbits (0.1 mg RP) and rats (0.05 mg RP). Initial immunization was performed with Freund's complete adjuvant followed by booster immunizations with Freund's incomplete adjuvant at days 21, 35, 49 and 63 and then monthly. Animals were bled seven days after each boost.

#### H. Surface Iodination, Trypsinization, Sequential Extraction And Immunoprecipitation

Mature intact PE were enriched to >90% by the percoll-sorbitol method (Kutner et al., *J. Cell. Physiol.* (1985) 125:521-527) after initial disruption of rosettes (Handunnetti et al., *supra*. Fifty or 100  $\mu$ l of PE were iodinated by the lactoperoxidase method using 1-2.5 mCi or 5 mCi of  $\text{Na}^{125}\text{I}$  (Amersham) respectively, and sequentially extracted with 1% (w/v) Triton X-100 followed by 2% (W/v) SDS (van Schravendijk et al.). For trypsinization, iodinated PE were incubated at 10% hematocrit with 10  $\mu$ g/ml of trypsin-TPCK (Sigma, St. Louis, Missouri) in PBS for 5-10 minutes at 21°C. Trypsinization was terminated by adding an equal volume of 200  $\mu$ g/ml soybean trypsin inhibitor type I-S (Sigma, St. Louis, Missouri) in PBS. The trypsinized cells were sequentially extracted as above. Immunoprecipitation used 5-7  $\mu$ l of  $^{125}\text{I}$ -SDS extract, 10-15  $\mu$ l of  $^{125}\text{I}$ -Triton X-100 extract, or 15-25  $\mu$ l of tryptic supernatant from surface iodinated cells, reconstituted in 500  $\mu$ l of 50 mM Tris, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100 pH 8.0 (NETT buffer) containing 1% BSA (Clinical grade, ICN Biomedicals, Irvine, CA) and a cocktail of protease inhibitors (1 mM Pefabloc SC, Boehringer Mannheim Biochemicals, Indianapolis, IN), 1 mM pepstatin A, 10  $\mu$ g/ml each of benzamidine, leupeptin and aprotinin, 3.7  $\mu$ g/ml of N-tosyl L-lysine chloromethylketone and N-tosyl L-phenylalanine chloromethylketone (Sigma, St. Louis, Missouri)). The reconstituted extracts were incubated overnight with <10  $\mu$ l of sera and processed as previously described (Van Schravendijk et al., *supra*). For depletion of anti PfEMP1 antibodies with PfEMP1-RP, 7.5  $\mu$ l of sera were incubated, 3 h 21°C, in 500  $\mu$ l of NETT-BSA containing 15  $\mu$ g of RP bound to glutathione-Sepharose 4B beads. The beads were centrifuged and removed, 5  $\mu$ l of  $^{125}\text{I}$ -SDS extract added and immunoprecipitation performed.

#### I. Western Blots

Trophozoite-stage PE were extracted sequentially with 1% Triton X-100 and 2% SDS to a final concentration of  $10^8$  parasites per ml. 2.5  $\mu$ l of extract was SDS-PAGE

fractionated on 5% acrylamide gels, transferred onto Immobilon-P membrane (Millipore Corp., Bedford, MA) and immunoblotted using an ECL Western blotting protocol (Amersham Int., Buckinghamshire, England). Membranes were incubated overnight at 5°C in 50 mM Tris, 150 mM NaCl, 0.1% Tween 20 pH 8.0 (TBS-T) containing 10% w/v nonfat dry milk, followed by 1 h incubation with the primary Ab diluted in TBS-T, 5% w/v dry milk (TTM), one wash with TBS-T, two with high-salt TTM (0.5 M NaCl) and two with TTM. HRP-conjugated donkey anti Rabbit IgG (Jackson ImmunoResearch Labs. Inc.) was diluted 1:50,000 in TTM and added to the membranes for 45 minutes. The membranes were washed once with TBS-T, twice with high-salt TTM, three times with TTM containing 0.5% Triton X-100 and three times with TBS-T.

#### J. PE Agglutination

Agglutination assays were performed with modifications as described earlier (Aguiar et al., *Am. J. Trop. Med. Hyg.* (1992) 47:621-632). PE were washed twice with RPMI-1640, 25 mM HEPES, 1% BSA pH 6.8 or 7.2 (binding media (BM)) and resuspended to 20% hematocrit. 15 µl of blood was mixed with an equal volume of diluted sera, incubated 45 minutes at 37°C, in a G24 environmental incubator shaker (New Brunswick Scientific) with continuous rotation (125 rpm) examined microscopically and scored based on the size and number of the specific PE agglutinates.

#### K. Soluble Receptors And Cytoadherence Microassay

Soluble CD36 was obtained in the form of harvest supernatant by cleaving phosphoinositol glycan-linked CD36 from the surface of stable transfected CHO cells using PI-PLC (Lin et al., *Science* (1990) 249:677-679) and stored at 5°C. The CD36 concentration in the harvest supt. was approx. 1-2 µg/ml. Purified TSP was purchased from Gibco BRL. A modification of the standard microscopic adherence microassay (Hasler et al., *Am. J. Trop. Med. Hyg.* (1993) 48:332-347) was used for antibody-mediated inhibition of PE adherence. 7 µl of MAb 179 at 50 µg/ml in PBS was used to coat each well (1hr, 21°C), washed once with 50 µl of PBS and blocked, 30 minutes, 21°C with PBS containing 1% BSA. The blocking solution was

washed twice with PBS and 50  $\mu$ l of the appropriate amount (usually 0.2, 0.4 or 2  $\mu$ g/ml) of sCD36 added and incubated 21°C for 1 hr. TSP at 50  $\mu$ g /ml was coated directly on the plastic (2 h, 21°C). Each well was washed twice with BM. PE were washed once with BM and resuspended to 4% hematocrit in BM +10% FCS. An equal volume of diluted serum was added and the cells incubated with the sera for 1 h. at 37°C. 50  $\mu$ l of cells at 2% hematocrit were added to each well and incubated 1 h at 37°C. After removing the parafilm mask, the plates were washed four times with BM, the cells fixed, strained and counted (Hasler et al.). For determination of adherence of recombinant proteins, the recombinant proteins were preincubated for 1 hour with the immobilized receptor before PE were added. Reversal of PE adherence to CD36 was performed by allowing 30 minute adherence with PE followed by 3 washes with BM and addition of BM containing antibodies or recombinant protein for 45 minutes, followed by two washes, fixing and staining, as described above.

L. Confocal Fluorescence Imaging Microscopy

Immunofluorescence microscopy was performed with a Bio-Rad MRC-600 system (Bio-Rad Laboratories, Cambridge, MA) interfaced to an Olympus IMT-2 inverted microscope as described previously (Gormley et al., *J. Cell. Biol.* (1992) 119:1481-1495).  $1 \times 10^8$  cells (P=4%, trophozoite and schizont stages) were washed three times in RPMI-1640 (RPMI), resuspended in 10  $\mu$ l of serum and incubated, 2 h, 37°C, with constant shaking. The samples were washed three times with RPMI and Rhodamine (TRITC)-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc.) added at a 1:10 dilution in RPMI for 30 minutes, 37°C. The cells were washed three times in RPMI, diluted to 0.3% hematocrit and viewed in a Dvorak chamber.

M. Immunoelectron Microscopy

Cells (5% hematocrit P=5%) were incubated in RPMI containing 2% BSA, 30 minutes, 25°C with constant shaking. Primary rat serum was added at 1:100 dilution for 1 h, at 25°C with constant shaking, followed by three washes with RPMI. 5 nm gold conjugated goat anti-rat IgG (Goldmark Biologicals)

was incubated with the cells at a 1:50 dilution in RPMI, 30 minutes, 25°C. The cells were washed three times with RPMI, fixed overnight at 4°C in 2% glutaraldehyde, 1% tannic acid, 4% sucrose, 0.1 M phosphate buffer pH 7.4, washed with 0.1 M phosphate buffer and post-fixed in 2% osmium tetroxide in 0.05 M phosphate buffer (pH 7.4) on ice for 90 minutes. The specimens were washed four times with deionized water and incubated with 1% uranyl acetate for 15 minutes at 25°C, washed four times with deionized water, embedded in 2% agarose and dehydrated in graded steps of acetone. The cells were infiltrated and embedded in Spurr's. Thin sections were cut on a Reichert-Jung Ultracut E ultramicrotome and post-stained with 2% uranyl acetate and 1% lead citrate. The sections were examined with Hitachi H-7000 STEM.

N. Preparation of rC1-2 Fragments

1. Oligonucleotide Primers

Oligonucleotide primers were synthesized based upon the sequence of PfEMP1. The name of the primer represents the position of the amino acid coded at the 5' or 3' end of the oligonucleotide. Oligonucleotides from the coding strand of rC1-2 (a.a. 576-808 of PfEMP1) were as follows:

1: AAGGAAGACAAAATTATGTCCTAT  
25: AATGGAGAGACGAACATGG  
53: TCCAAAAATGGGTTGAACAAAA  
80: ATTGGACTCATGATGATTTTC  
96: TTTTGGAAATTATTCAGGATACT  
135: CTAAAGGTTTTGTCGCTGAAA  
156: AAGCCGATAAATGCCTAAAAAC

Oligonucleotides from the noncoding strand were as follows:

59: TTTTTGTTCAACCCATTTTTGGA  
87: TGAAGAAAATCATCATGAGTCCA  
102: AGTATCCTGAATAATTTCCAA  
140: TTCAGCGACAAAACCTTTAGT  
179: GAGCGGGCGACACTTCTATCT

192: CTTAGGGTCGGCAGGTGGTG

233: ATCCGTCTTTTCCTCCTGGACTT

5 Oligonucleotides from the coding strand (5' oligos) carried a BamH1 site and oligonucleotides from the non-coding strand (3' oligos) carried an EcoRI site at their 5' ends.

## 2. RT-PCR Protocol

10 Total RNA from late-ring stages of *P. falciparum* was isolated as described before (Pasloske et al. 1993) or using Catrimox-14 (Iowa Biotechnology Corp.) and lithium chloride precipitation according to the manufacturers instructions. The RNA was Dnase-1 treated to remove all contaminating DNA. Complete removal of DNA was verified by PCR reaction using  
15 various sets of primers (no PCR product). Usually, 1 µg of RNA was treated with 1 unit of Dnase-1 (promega) for 15 min at 21°C, followed by the addition of 2 mM EDTA and 15 min. incubation at 65°C, phenol chloroform extraction and ethanol precipitation. 1-2 µg of treated RNA was reversed transcribed  
20 in volume of 30 µl with 0.5-1 µM of the C1-2 179-EcoRI primer containing an EcoRI restriction site. C1-2 primer sequence: (cgg aat tct g)GAG CGG GCG ACA CTT CTA TCT (with the EcoRI restriction site indicated in lower case). The RNA was heat denatured at 70°C for 10-15 min in the presence of the C1-2  
25 179-EcoRI primer and cooled on ice (1-2 min). 0.1 M DTT, RT buffer, 30-40 units of RNasin and 0.5M dNTPs added and the mix was equilibrated at 50°C for 2 min before addition of 300 units of superscript RT (Gibco BRL) and 1h incubation at 50°C. The RNA template was removed from the first strand cDNA  
30 by 15 min. incubation at 37°C with 2-3 units of Rnase H (Gibco BRL) and purified with glass max purification system (Gibco BRL). The purified first strand cDNA was subjected to PCR as above with the C1-2 179-EcoRI and the C1-2 1-BamH1 [(cgc gga tcc) AAG GAA GAC AAA ATT ATG TCC TAT (with the BamHI site in  
35 lower case)] primer set, only the 1 min 42°C incubation was replaced with 1 min at 50°C. To ensure that the product is RNA derived a mock RT reaction (no enzyme) was performed. Non-DNase RNA and gDNA were used as additional controls. Th



PCR products were cloned into the pGEX-3X vector sequenced and tested for protein expression as above.

### 3. Universal Degenerate Oligonucleotide Primers

Degenerate oligonucleotides were prepared for use as universal primers to PCR the corresponding regions from gDNA of different *P. falciparum* strains. The 5' forward primer included a Bam H1 site and the 3' primers carried a EcoRI site for direct cloning into the pGEX-3X vector as described above (See discussion of GST fusion proteins). From the sequences of a number of *P. falciparum* strains, universal degenerate primer sequences were identified as follows:

Uni179-5'

5'	TTT	TTT	TGG	GAG	TGG	GTA	TAT	GAT	ATG	TTA
				ATT			T	ACC	C	A
							C			
AA:	F	F	W	M	W	V	H	D	M	L
				K			T	E		
				D			Y	H		

Uni179-3'

3'	ACC	ACA	ATT	GAT	AAA	TTA	CTA	CAA	CAC	GAA
	T				A	G	C	A	T	
AA:	T	T	I	D	K	L	L	Q	H	E
						I		N		

PCR amplification using these universal primer compositions was performed with 50-100 ng of DNA, 0.5  $\mu$ M of each primer, 2.5 units of Taq enzyme, 200  $\mu$ M dNTPs in 50  $\mu$ l volume. Initial denaturation 2 min at 94°C followed by 30 cycles of 50 sec at 94°C, 1 min at 42°C, 90 sec at 72 C and final extension at 72°C for 10 min. The products were gel isolated (Wizard™ PCR DNA isolation kit, Promega), digested with BamH1 and EcoRI, cloned into pGEX-3X vector as described and sequenced.

### 4. Preparation of Fragments of rC1-2[1-233] and Mutant Fusion Proteins of rC1-2[1-233]

The preparation, production and purification of the GST fusion protein rC1-2 (233 amino acids from positions 576-808 of the MC PfEMP1) denoted rC1-2[1-233], was carried out as described in Section F, above. Recombinant protein fragments

of rC1-2[1-233]; rC1-2[1-192], rC1-2[1-179], rC1-2[1-140], rC1-2[1-102], rC1-2[1-87], rC1-2[1-59], rC1-2[11-179], rC1-2[25-179], rC1-2[53-192], rC1-2[80-192], rC1-2[96-192], rC1-2[135-192], rC1-2[156-192] and rC1-2[156-233] were generated using the above described primers. Cysteines at various positions were replaced by using a primer sequence with a Serine codon in place of the corresponding Cysteine codon, generating the following mutant fragments rC1-2[1-179] Ser<sup>159</sup>, rC1-2[1-179] Ser<sup>168</sup>, rC1-2[1-179]Ser<sup>159</sup> and Ser<sup>168</sup>, rC1-2[1-179] Ser<sup>45</sup> and rC1-2[1-179] Ser<sup>49</sup> recombinant protein with a serine substitution of each or both cysteines. All mutants were tested for expression, and produced similar amounts of recombinant protein as determined by SDS-PAGE stained with coomassie blue. Oligonucleotides from the above, particularly those corresponding to the 1-179 region, were used to generate a PCR product from 10 different strains of *P. falciparum*, and GST-fusion proteins were prepared.

O. Binding of CHO Cells to GST-PfEMP1 Fusion Protein, Immobilized on Plastic

7  $\mu$ l of 50  $\mu$ g/ml of MAb 141.4 (Mouse IgG, anti-GST) in PBS at pH 7.4 was spotted on parafilm masked petri dish for 1 hour at 21°C. The dish was washed twice with RPMI-1640, at pH 7.3 containing 1% BSA (clinical grade, ICN, Irvine CA), and incubated with RPMI + 1% BSA for 30 minutes at 21°C. The dish was then washed twice with RPMI + 0.05% BSA. Purified fusion proteins (50-200  $\mu$ g/ml in PBS) were added to the dish and incubated for 1 hour at 21°C. The dish was again washed twice with RPMI + 0.05% BSA. 50  $\mu$ l of approximately  $2 \times 10^6$  cells/ml (CHO, CHO-CD36 or CHO-ICAM cells) in RPMI + 0.05% BSA were incubated with the immobilized fusion protein for 1 hour at 37°C. The dish was then washed four times, then fixed, stained and counted. The results are presented as cells bound/mm<sup>2</sup> +/- standard dev.

P. Blockade of PE Adherence with Recombinant Protein Derived from PfEMP1

30  $\mu$ l of recombinant protein (RP) in PBS (approx. 100  $\mu$ g/ml, or as indicated) were incubated for 1 hour at 21°C with CD36 (0.2  $\mu$ g/ml), immobilized to plastic as described

above, then aspirated. 50  $\mu$ l of parasitized erythrocytes (PE) (P=6-10%) at 2% hematocrit in RPMI, 25 mM HEPES, 1 % BSA, pH 6.8 (BM) were added to the immobilized protein and incubated for 1 hour at 37°C, washed four times with BM, fixed stained and counted as above. Reversal of PE adherence to CD36 was performed substantially as described in Section K, above.

Q. Receptor/Counter Receptor Precipitation Assay (RCPR) and Analysis of Mutant Recombinant Fusion Proteins

25  $\mu$ l of GammaBind™ Plus Sepharose resin (Pharmacia LKB BioTech., Uppsala, Sweden) was coated with 15  $\mu$ g of MAb 141.4 for 1 hour at 21°C. The resin was washed three times with PBS and resuspended in 1 ml of BM. 5 $\mu$ g of purified GST-fusion protein, or 50  $\mu$ l of lysate from bacteria expressing the GST fusion protein was added, and the mixture was incubated for 2 hours at 21°C with rotation. The resin was washed three times with PBS and resuspended in 400 $\mu$ l of BM (or BM containing 1 mM  $\text{Ca}^{2+}$  at pH 7.3, for thrombospondin (TSP) experiments). 100 $\mu$ l of sCD36, other pig-tailed receptors (approx. 1-5  $\mu$ g/ml) or human TSP (Gibco-BRL, 20  $\mu$ g/ml) was added and incubated for 2.5 hours at 21°C, with rotation. The resin was washed twice with 1 ml of BM, once with 1 ml BM without BSA, and then solubilized with 40  $\mu$ l of 5% SDS sample buffer. 5 or 10  $\mu$ l of the solubilized sample were fractionated by SDS-PAGE on a 10% acrylamide gel (5% for TSP) and ECL western blot as described. 1.25  $\mu$ g/ml of biotinylated-MAb 179 or rabbit serum 186 (anti-TSP), diluted 1:5000 were used to detect bound pig-tail receptors, or bound TSP, followed by secondary HRP-conjugated streptavidin or HRP-conjugated donkey anti rabbit IgG (Jackson ImmunoResearch Labs, Inc., West Grove, PA).

R. Preparation of Chimeric rC1-2 [1-179] GST Fusion Proteins

Chimeric protein composed of the 5' portion of the rC1-2 [1-179] sequence of one *P. falciparum* strain and a 3' portion derived from the sequence of a different strain were prepared by taking advantage of a unique Mfe-1 restriction site (CAATTG) present in the sequence of rC1-2 [1-179] of different strains. The site resides at position 433 in strain

MC R+, position 418 in ItG2-F6, 376 in clone FCR3-C5 and position 439 of HB3 (except for the sequence of MC R+, all sequences were obtained from gDNA). Cl-2 [1-179] clones (in pGEX-3X vector) of the different strains were digested with BamH1 and Mfe-1 and the different parts (BamH1-MfeI 5' part of the sequence and the remaining 3' part pGEX-3X fragment) and purified from agarose gel. The 5' fragment of ItG2-F6, HB3 and FCR3-C5 were ligated to the 3'-pGEX fragment of MC R+ and the 5' fragment of MC R+ was ligated to the 3'-pGEX fragment of ItG2-F6, HB3 and FCR3-C5 thus creating six chimeric constructs: ItG2-F6/MC R+, FCR3-C5/MC R+, HB3/MC R+, MC R+/ItG2-F6, MC R+/FCR3-C5 and MC R+/HB3. The ligation products were transformed into DH5 $\alpha$  cells, sequenced and screened for protein expression. The chimeric fusion proteins were assayed by the "ECL" RCRP method as described above. Reversal of PE adherence to CD36 was performed by allowing 30 minute adherence with PE followed by 3 washes with BM and addition of BM containing antibodies or recombinant protein for 45 minutes, followed by two washes, fixing and staining, as described above.

#### Example 2- Identification of cDNA

Previously, the preparation of a rabbit serum, 05-75, which reacted with two very large (>300 kD) malarial proteins, PfEMP1 and PfEMP3, associated with the erythrocyte membrane of *P. falciparum* PE was described (van Schravendijk et al., supra). Adsorption of serum 05-75 against a recombinant protein (RP) corresponding to part of PfEMP3 ( $\beta$ -gal-12.1.3), ablated immunoreactivity with PfEMP3 without effect on immunoprecipitation of <sup>125</sup>I-PfEMP1 (van Schravendijk et al., supra). Screening of a gDNA  $\lambda$ gt11 expression library from MC parasites with 05-75 serum preadsorbed with the  $\lambda$ 12.1.3 clone identified one insert that hybridized to a large (8-10 kb) mRNA band of size sufficient to encode a very large protein. This 1.8 kb insert, denoted A62, gave maximal signal intensity with RNA from lat ring stage parasites. RP derived from part of clone A62 blocked immunoprecipitation of MC PfEMP1 by serum 05-75. In view of these results this gDNA

clone was explored further. A cDNA library of K+ MC strain parasites was subjected to PCR with primers derived from A62 to generate cDNA clones extending 5'. One of these clones, of 1.1 kb, included sequences corresponding to the 3' end of clone D1, but proved to be highly unstable in *E. coli*. Nevertheless, repeated PCR using diverse probes from this clone identified a more stable cDNA, denoted D1 (Figure 1). From D1 additional contiguous cDNA clones were produced, extending 5', designated A1-C2 (Figure 1). Clones D2 and D3 were also identified by PCR with this cDNA library (Figure 1). D2 overlapped D1 completely whereas D3 diverged from the sequence shared by D1 and D2 in several places (Figure 2). Repeated attempts to identify stable overlapping cDNAs that extend 3' from D1 or D2 were unsuccessful. However, the sequence was extended 3' from D1 (and D2) by PCR cloning from the MC parasite gDNA, extending the sequence to 8227 nucleotides (Figure 1, F-gDNA). Further 3' extension, generated the cDNA clone G1, extending this contiguous sequence to 9159 nucleotides (Figure 1). The cDNA sequence was extended further 3' from D3 to identify clone E1 (Figure 1). Both sequences are of full length and show the extracellular domain of the MC-PfEMP1. The deduced amino acid sequence of these variants is shown in Figure 2 and 12.

### Example 3- Structure Of The Gene and Variant Sequences

Clone A1 included 244 nucleotides before a start codon and initiation of a single open reading frame which extended through overlapping cDNA clones via D3 to the 3' end of E1 (5186 nucleotides, MC var 2 Figure 1), or to clone G1 via D2 and the F-g DNA clone (9159 nucleotides, MC var 1 Figure 1). PCR products were generated corresponding to clones B1-F-gDNA and A1-D1/D2 of MC var-1, and clones B1-E1 and A1-D3 of MC var-2, thus identifying var-1 and var-2 as two independent PfEMP1 genes of MC strain parasites. These genes have overlapping sequence with a single amino acid substitution between cDNA clones D2 and D3 up to amino acid 871 at which point their sequences diverged (Figure 2). A 725 bp intron was identified at nucleotide 7429 by comparison

with sequence data obtained for other genes in the same family described in an accompanying report, giving a deduced amino acid sequence of 2924 residues (Figure 12). Small differences in nucleotide sequence and deduced amino acid sequences were observed in several of the overlapping cDNA clones (Figure 2).

A single, putative transmembrane domain (amino acids 2450-2475) was identified just before the putative splice junction, followed by a presumed intracellular domain encoded by the 3' exon. The majority of the protein sequence is therefore extracellular (amino acids 1-2449, Figure 2), with a relatively short intracellular domain (~440 amino acids). This is consistent with the combined size of tryptic fragments of MC PfEMP1 (210 kDa) generated from treatment of intact PE. The 2574 amino acid sequence from MC strain parasites shows extensive homology with the several sequences in a gene family. Analysis of the Swiss-Prot, NBRF-PIR and Translated GenBank (release 86) databases identifies the sequence as novel. There are 18 potential N-linked glycosylation sites. A RGD motif (amino acids 1212-1214) and a LDV motif (amino acids 142-144) that could be involved in protein-protein adhesion are evident in the sequence (Figure 2). The deduced amino acid sequence lacked stretches of tandem repeats.

Analysis of the distribution of the 105 cysteines and other residues (Figure 2) revealed 4 domains, denoted DBL-1 through DBL 4, homologous with the Duffy antigen Binding Ligand (DBL) domains of *P. vivax*, *P. falciparum* and *P. knowlesi*, each containing 5 consensus motifs rich in cysteine residues (Figure 2). Between the DBL domains there are 3 examples of another cysteine rich motif (CRM) with 3 cysteines in a 7 amino acid stretch and additional homology over approx. 45 amino acids. These domains, denoted CRM-1, CRM-2, CRM-3, share the sequence CNXKCXCX<sub>2</sub>K and are located between the different DBLs (Figure 2). CRM-1 and CRM-2 are more closely related than CRM-3, sharing a longer motif, CX<sub>3</sub>CX<sub>3</sub>CXC, and other residues over 38-42 amino acids (Figure 3). Although there are 6 cysteine residues between DBL-3 and DBL-4, a CRM was absent from this region.

Southern blotting of fragments of the novel genes with Eco RI or Eco RI plus Hind III digested gDNA was performed with DNA from K+C+ and K-C- MC strain parasites together with DNA from 6 other parasites of widely dispersed geographic origin and diverse adherence phenotypes (Figure 4 and Table 1). The A1 fragment from the 5' end of the gene (-244-518) hybridized to DNA from all eight parasites, yielding 5-8 hybridization-positive fragments of similar size. Similar results were obtained with 3 other fragments from bp 3226-6446 (fragments E1, F-a, F-b, Table 2). In contrast, fragments derived from bp 455-3768 and the 3' end (6692-8227) did not hybridize with DNA from all parasites, reacting almost exclusively with MC parasites (Figure 4B and Table 1). Hence, the novel gene shares extreme 5' sequence and the region 3644-6446 with gDNA of diverse parasites, while the central region (nucleotides 455 to 3768) and the 3' end are significantly different between MC and other parasites. Since there is only a single Eco RI site, within fragment F, and no Hind III sites in the sequence, the presence of multiple fragments with MC K+C+ parasites indicates multiple forms of the novel gene. This is consistent with significant sequence differences in independent cDNA clones from the same parasite.

Table 2

Southern blot hybridization of DNA clones from the MC PfEMP1 gene with EcoRI digested DNA of different *P. falciparum* parasites.

<u><i>P. falciparum</i> Strain/Clone</u>								
Frag. #	MC K+	MC K-	Dd2	FCR3-C5	FCR3-C6	ItG2-ICAM	ItG2-G1	PALO ALTO
A1	+	+	+	+	+	+	+	+
B1	+	+	-	-	-	+	+	-
C1	+	+	-	-	-	-	-	-
D1	+	+	-	-	-	-	-	-
D2	+	+	+	-	-	-	-	-
D3	+	+	-	-	-	-	-	-
E1	+	+	+	+	+	+	+	+
F-a	+	+	+	+	+	+	+	-
F-b	+	+	+	+	+	+	+	+
F-c	+	+	+	-	-	+	-	-
G1	+	+	+	+	+	+	+	+

\* Weak hybridization

† Multiple bands

# DNA fragments A1 - G1 are defined in Figure 1. Fragments F-a, F-b, and F-c correspond to bp 3226-5139, 5140-6446 and 6692-8227 in the map (Figure 1).

Southern blot hybridization was performed as in Figure 4 and experimental procedures.

#### Example 4- Antibodies Generated Against Recombinant Fusion Proteins Recognize PfEMP1

To identify the gene corresponding to the novel cDNA, laboratory animals were immunized with RP corresponding to different parts of the cDNA. A number of recombinant proteins were expressed as GST or MBP-fusions in *E. coli* (Figure 1). The RP (rA1[3-158], rB1[161-385], rC1-1[402-605], rC1-2[576-808], rC1-2[1-179]-MC, rC1-2[1-179]-"ItG", rC1-2[1-179]-"HB3", rD1[818-1003], rD2[982-1320], rF1-1[1300-1707], rF1-3[2171-2450], rG1[2550-2794], rD3[992-1243], rE1-1[1219-1471], rE1-2[1454-1719]) were readily purified and used for



immunization in either rats, rabbits or goats (See Tables 8 and 9, below).

Sera were screened for immunoprecipitation of  $^{125}\text{I}$ -protein from SDS extracts of PE containing mature asexual stages of MC K+ parasites, immunoprecipitation of labeled fragments cleaved from the surface of iodinated PE (MC K+) by mild trypsinization (Figure 23). A high molecular weight  $^{125}\text{I}$ -protein was immunoprecipitated by sera from several animals immunized with rC1-2 but not by pre-immune sera (Figure 5A). Rat anti-rC1-2 antibodies immunoprecipitate a 90 kd tryptic fragment (TF90) also immunoprecipitated with Aotus anti MC specific sera. The same fragment was affinity purified with immobilized CD36 but not by other immobilized host receptors. The fragment TF125 affinity purified with TSP was immunoprecipitated by antibodies to rD1, rF1-1 and rF1-3 (See Table 9 and Figure 23). This is a further indication that the TSP binding domain may be contained on the region corresponding to these protein fragments. Two rabbits immunized with rD1 also produced immunoprecipitating antibodies (Figure 5B and Table 9). Sera from two rabbits immunized with rB1 failed to immunoprecipitate  $^{125}\text{I}$ -labeled proteins.

The properties of the  $^{125}\text{I}$ -protein identified by the anti rC1-2 and rD1 sera were identical to those of  $^{125}\text{I}$ -PfEMP1 (Figure 5C). The  $^{125}\text{I}$ -protein co-migrated with  $^{125}\text{I}$ -PfEMP1 immunoprecipitated by three critical sera: a pool of human immune serum that agglutinates MC K+ PE; Aotus anti-MC K+ serum that specifically agglutinates and immunoprecipitates  $^{125}\text{I}$ -PfEMP1 from this strain (Howard et al., 1988) and, rabbit 05-75 serum that immunoprecipitates MC strain  $^{125}\text{I}$ -PfEMP1 (van Schravendijk et al., supra). The  $^{125}\text{I}$ -protein was not immunoprecipitated from the Triton X-100 extract of  $^{125}\text{I}$ -labeled MC PE and was destroyed by treatment of intact PE with trypsin, additional properties which define  $^{125}\text{I}$ -PfEMP1 (Figure 5C).

The anti-rC1-2 and rD1 sera did not immunoprecipitate  $^{125}\text{I}$ -PfEMP1 from ItG2-ICAM parasites, even though a  $^{125}\text{I}$ -PfEMP1 was immunoprecipitated by the pool d

human sera. Hence, the anti-rC1-2 and anti-rD1 sera define MC K+ strain-specific epitope(s) on  $^{125}\text{I}$ -PfEMP1, similar to sera from Aotus monkeys infected with this parasite (Howard et al., 1988, *supra*).

5 To address the possibility that immunoprecipitating antibodies elicited by rC1-2 and rD1 immunization cross react or result from spurious cross-reactivity, anti-rC1-2 and anti-rD1 sera were preadsorbed with different GST-fusion RP and immunoprecipitation of  $^{125}\text{I}$ -PfEMP1 repeated. Preadsorption  
10 of anti-rC1-2 serum with glutathione-Sepharose beads bearing rC1-2 completely ablated the capacity of this serum to immunoprecipitate  $^{125}\text{I}$ -PfEMP1, while beads bearing rD1, rB1, rA62-5, rPfEMP3 or GST itself had no effect on the immunoprecipitation (Figure 5D). In contrast, preadsorption  
15 of anti-rD1 serum with beads bearing rC1-2 had no effect on immunoprecipitation, while in this case preadsorption with rD1 completely ablated immunoprecipitation (Figure 5E). This demonstrates that elicitation of the anti-PfEMP1 antibodies by these two RP does not reflect cross reactivity and that GST  
20 per se does not involve the elicitation of the anti-PfEMP1 antibodies. The anti-rC1-2 and anti-rD1 sera independently define structural similarity between each RP and two regions of the MC K+ PfEMP1. Hence, the cDNA corresponds to part of the PfEMP1 gene of the MC strain of *P. falciparum*.

25 None of the sera precipitated  $^{125}\text{I}$ -PfEMP1 from SDS or Triton X-100 extracts of surface iodinated PE from MC K-parasites. Since  $^{125}\text{I}$ -PfEMP1 is absent from the surface of these non-adherent PE (Aley et al., *J. Exp. Med.* (1984) 160:1585-1590), these results are consistent with the identity  
30 of the novel gene as PfEMP1.

Rabbit sera against rC1-2 were tested for reactivity on Western blotting with proteins in SDS extracts of different *P. falciparum* strains and clones (Figure 6). This panel of parasites included examples with known differences in the size  
35 and antigenic specificity of their  $^{125}\text{I}$ -labeled PfEMP1 proteins as defined by immunoprecipitation with strain-specific Aotus sera. A high molecular weight, size variant antigen was identified in many of the parasite samples

but was missing from the uninfected human erythrocyte sample (NRBC in Figure 6). The erythrocyte spectrin bands were immunoreactive with all parasites and with uninfected erythrocytes. The pre-bleed of this rabbit sera was also reactive to spectrin but did not recognize the size-variant bands. Low level cross-reactivity with Histidine rich protein 1 (HRP1) was also detected (Figure 6). Several pieces of evidence lead to the conclusion that the size-variant antigens (200 to >300 kDa) identified by the rabbit anti-rC1-2 sera are the PfEMP1 proteins of these parasites. First, the MC K+ sample exhibited a reactive band, whereas the MC K- sample exhibited little or no reactivity. Second, FVO strain showed a reactive band at approximately 205 kDa, migrating just faster than the spectrin 1.2 band. A similar size <sup>125</sup>I-PfEMP1 protein is detected with this parasite (van Schravendijk et al., *Blood* (1991) 78:226-236). Third, trypsinization of intact MC K+ PE reduced the intensity of the immunoreactive band in a manner that was dependent on the dose and time of trypsinization. Fourth, FCR-3 parasite clones exhibited 2 or 3 reactive bands absent from uninfected erythrocytes with mobilities characteristic of clone C5 <sup>125</sup>I-PfEMP1. ItG2G1, HB2, D10 and 7G8 parasites each possessed a single reactive band but with different apparent molecular weights, while ItG2-ICAM, Dd2 and PA parasites did not display a reactive band. Although the immunoreactive PfEMP1 proteins share epitopes recognized by antibodies raised against the rC1-2 portion of MC strain PfEMP1, no hybridization of the C1 DNA fragment to DNA from these parasites was found. Other parasites, including some known to possess a different form of PfEMP1, lack epitopes defined by antibodies raised against this part of the MC PfEMP1.

Example 5- Anti-PfEMP1 Antibodies React With The Surface Of PE In a Strain-Specific Manner

Rabbit and rat anti-rC1-2 sera were tested by confocal fluorescence microscopy for reaction with the surface of intact, non-fixed PE. Neither of the two rabbit anti-rC1-2 sera tested were positive with MC strain PE. However, each of

the f ur rat sera reacted by indirect immunofluorescence with the surface of MC PE (Figure 7, panels A and B, Table 2). Th immunofluorescence reactivity was distributed in a speckled pattern over the entire PE surface. Fluorescence staining was observed on ~80% of late-trophozoite and schizont PE.

Preimmune rat sera or rat sera to PfEMP3 (van Schravendijk et al., 1993, *supra*) were not reactive. Uninfected erythrocytes and erythrocytes infected with young trophozoites or ring stages were not labeled. No reactivity was found with MC K-, ItG2-ICAM, ItG2-G1 or FCR<sub>3</sub>/C5 PE.

Sera raised against the RP listed in Example 4 and Table 9 were screened for antibody-mediated agglutination of intact PE.

Two of the four rabbit sera against rC1-2 mediated agglutination (dilution 1:5) of MC strain PE, after disruption of rosettes. 3 out of four rat anti-rD1 sera agglutinated MC strain PE, after rosettes were disrupted. No agglutination was detected with rabbit anti-rD1 sera. Each of the four rat sera against rC1-2 mediated specific PE agglutination (Table 2). Agglutinates of several to hundreds of intact, mature PE were formed after incubation of infected blood with rat anti-rC1-2 sera (Figure 8). These agglutinates closely resembled those seen with Aotus anti-MC serum (Figure 8). Each of the rat sera collected after the first immunization with rC1-2 were agglutinating with a titer of >1:10. The extent of agglutination and titer increased after subsequent immunizations, with titers of 1:10 to >1:100 (Table 3). Uninfected erythrocytes were not present in these agglutinates, nor were preimmune sera reactive.

PE from other parasites known to express an antigenically different form of PfEMP1, ItG2-ICAM and Palo Alto ("PA") were not agglutinated (Figure 8 & Table 3), although they were agglutinated by pooled human immune sera. MC K- PE were not agglutinated (Figure 8 & Table 3). The initial failure of ItG2-ICAM and MC K- PE to be agglutinated by the anti-rC1-2 sera correlates with the failure of such sera to immunoprecipitate <sup>125</sup>I-PfEMP1 from these parasites. However, later bleeds (e.g., >day 195) of rat #1 anti rC1-2

and at least one of rat anti-rD1 agglutinated PE of different strains. These sera agglutinated PE of strain MC R+, ItG2-F6 and ItG2-ICAM, with some cross-reactivity with strain Palo Alto (See Table 9), but did not agglutinate PE of strains MC R-, ItG2-G1 or Dd2. These results indicate that PfEMP1 contains antigenic epitopes that are cross reactive in some, but not all strains.

Table 3

Agglutination of PE with sera containing anti-PfEMP1 antibodies.

PARASITE STRAIN						
SERUM	MC K+		ItG-ICAM		MC K-	
	AGGL. <sup>a</sup>	TITER <sup>b</sup>	AGGL. <sup>a</sup>	TITER <sup>b</sup>	AGGL. <sup>a</sup>	TITER <sup>b</sup>
human immune pool	4+	125	4+	125	0	--
Aotus anti MCK+ (9050)	4+	125	0	--	0	--
Aotus anti MCK- (7925)	0	--	ND	ND	0	--
Rat #1 anti rC1-2	4+	>100	0 <sup>c</sup>	2+ <sup>d</sup>	0	--
Rat #2 anti rC1-2	3+	20	0	1+ <sup>d</sup>	0	--
Rat #3 anti rC1-2	1+	10	0	1+ <sup>d</sup>	0	--
Rat #4 anti rC1-2	3+	20	0	1+ <sup>d</sup>	0	--
Rats #1-4 prebleed	0	--	ND	ND	ND	ND

a. Semi-quantitative agglutination score at 1:5 serum dilution: 0, no agglutination; 1+, 10 or more agglutinates of <20 PE; 2+, 10 or more agglutinates of 20-50 PE; 3+, 10 or more agglutinates of 100-200 PE; 4+, 10 or more agglutinates >200 PE.

b. Reciprocal of maximum serum dilution at which PE agglutination was observed.

c. Few, very small agglutinates (less than 1+) were detected at dilution of 1:5

d. Agglutination score of sera of day >195.

ND: Not determined.

Rat sera tested, were found to be active from second immunization and with monthly boosters, were also reactive beyond day 200.

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Immunoelectronmicroscopy was performed to localize the reactivity of the anti-rC1-2 antibodies on the PE surface. Treatment of intact MC K+ PE with rat anti-rC1-2 antibodies followed by gold-conjugated goat anti-rat IgG yielded deposition of gold particles on the PE outer membrane (Figure 9). Up to 30% of schizont stage PE were positive. The deposition of gold particles was confined to knobs (Figure 9), with 50-70% of the knobs labeled. Gold particles were not deposited after treatment with control sera or rat anti-PfEMP3 serum. No binding was detected with ItG2-ICAM, MC K- PE or uninfected erythrocytes. PfEMP1, as defined by the anti-rC1-2 sera, is therefore localized on the surface membrane of PE at known protrusions.

Rat anti-rC1-2 antibodies react specifically with the surface of mature asexual PE. In all tests for surface reactivity, these antibodies react exclusively with MC stain K+ PE, congruent with immunoprecipitation of <sup>125</sup>I-PfEMP1 exclusively from these parasites.

Example 6- Anti-PfEMP1 Antibodies Block Adherence Of PE To CD36 But Not To TSP

To test serum-dependent inhibition of PE adherence, PE were preincubated with test serum before adding the mixture to plastic dishes coated with CD36 or TSP. Rabbit sera raised against rB1, rC1-2 and rD1 had no effect on PE adherence to TSP or CD36, even at 1:5 dilution (Table 4). Rat sera to rB1 and rD1 had no effect on PE adherence to CD36 or TSP. In contrast, each of the 4 rat sera raised against rC1-2 blocked adherence of MC K+ PE to CD36 but had no effect on adherence to TSP (Figure 10). In further experiments, rat and goat sera raised against rC1-2[576-808], and rat sera raised against rC1-2[1-179]-MC blocked PE adherence to CD36 (Table 9). The extent of inhibition at 1:5 serum dilution ranged from 15-60%. By comparison, Aotus anti MC K+ sera inhibited 15% dilution. In some experiments the preimmune rat sera had an inhibitory effect of 10-30% at 1:5 dilution, with <10% inhibition at dilutions of 1:10 or greater (Figure 11). The inhibitory effect of preimmune sera was eliminated or markedly reduced by dialysis. This had no effect on inhibition mediated by immune rat sera.

Each of the rat sera raised against rC1-2 inhibited adherence to CD36 in a dose-dependent manner. The results for the most potent rat serum (serum #1) (Figure 11) show blockade of adherence titratable to 1:100 dilution with significant inhibition over the preimmune control. At 1:10 dilution, dialyzed sera from three rats inhibited 27-64%, while dialyzed preimmune sera were without effect (Table 3). Another control rat serum, anti-PfEMP1, raised against an unrelated GST-malarial fusion protein, had no effect on adherence. None of the rat anti-rC1-2 sera, (dilution  $\geq$  1:10), blocked adherence of ItG2-ICAM, PA K-C+ or ItG2G1 PE to CD36. These results further demonstrate that the anti-rC1-2 are specific for interaction with the surface of MC K+ PE and support the idea that PfEMP1 mediates adherence of PE to CD36.

15     Example 7- Binding of CHO-CD36 cells to Recombinant rC1-2 Fusion Protein

Antibodies to rC1-2 block adherence of PE to CD36 in a strain specific manner, but do not effect the binding to thrombospondin, thus identifying rC1-2 as the possible binding domain of MC PfEMP1 for CD36. To test if recombinant protein rC1-2 mediate adherence to CD36, CHO, CHO-CD36 or CHO-ICAM cells were incubated with immobilized recombinant proteins derived from different parts of the MC PfEMP1 gene. CHO and CHO-ICAM cells did not bind to any of the RPs tested. CHO-CD36 cells bound specifically to rC1-2 (130 cells/mm<sup>2</sup>), but did not bind to rB1 or rD1 derived from the MC PfEMP1 gene, rA62-5 of the A62 clone or GST alone (See Figure 13). The binding of CHO-CD36 cells to rC1-2 was concentration dependent and reaches apparent saturation at RP concentrations of approx. 100  $\mu$ g/ml with a maximum binding of about 250 cells/mm<sup>2</sup> at 200  $\mu$ g/ml (see Figure 15). No binding of CHO or CHO-ICAM cells to rC1-2 was apparent at the highest concentration tested.

35     Example 8- Binding of CD36 to rC1-2

The binding of CD36 to different RP was tested with a modification of the RCPR assay, described above. GST-fusion proteins were immobilized to protein-G Sepharose beads, coated with anti-GST MAbs (MAb 141.4). The immobilized fusion proteins were

then incubated with the pig-tailed, soluble host cell receptors. Bound receptors were detected with MAb 179 using ECL western blotting. CD36 was affinity purified using immobilized rC1-2 and did not bind to immobilized rA1(3-158), rB1(161-385), rC1-1(402-5 605), rD1(818-1003), rF1-1(1300-1707), rF1-2(1688-2190), rF1-3(2171-2450), rG1(2550-2794) or the MCvar-2 specific recombinant proteins rD3(992-1243), rE1-1(1219-1471) and rE1-2(1454-1719) or to the RP fusion partner, GST.

Example 9- rC1-2 Blocks Adherence of PE to CD36

10           Antibodies to rC1-2 were shown to selectively block and reverse adherence of PE of the MC strain, in a strain specific manner. However, MAbs which bind CD36 have been shown to block all strains tested. As rC1-2 binds directly to CD36, it was then test d  
15           for its ability to block the binding of PE from other parasite strains.

          rC1-2 was preincubated with immobilized CD36 before the addition of PE containing the same concentration of RP. Four different parasite strains of diverse geographic origin, adherence phenotypes and knob expression were tested. These strains also  
20           express a serologically distinct PfEMP1 molecule as shown by agglutination and other assays, as described. At 100µg/ml RP, rC1-2 blocked adherence of PE from all four strains by upwards of 75-98% (See Figure 16). The four strains included in Figure 16 were MC R+ (solid bars), clone ItG2-ICAM (hatched bars), clone ItG2-G1 (grey  
25           bars) and clone Palo Alto K+ (open bars)). Other strains tested included MC R-, ItG2-F6, FCR<sub>3</sub>-C5, Palo Alto (K-) and Dd2. rC1-1 blocked adherence in each case, indicating that blockade of adherence by rC1-2 is not strain specific. None of the other RP tested had any effect on adherence to CD36 by PE of other strains,  
30           except for a small effect (15-20%) of rD1 on adherence of PE of strain ItG2-ICAM. Blockade of PE adherence to CD36 by rC1-2 was concentration dependent with an IC<sub>50</sub> (50% reduction in cell adherence) ranging from 0.3 µM (15 µg/ml) to 1 µM (53 µg/ml) (see Figure 18). Blockade of adherence of strain ItG-ICAM was almost  
35           identical to blockade of adherence of strain MC (see Figure 17). rC1-2[1-179] blocked adherence with an IC<sub>50</sub> of 0.78 µM (See Figure 24). These results demonstrate that although the CD36 binding region of different strains may be serologically distinct, they bind



the same region of CD36, and this binding may be blocked by rC1-2. rC1-2[1-179] reversed adherence of PE to CD36 with an approximate  $IC_{50}$  of 0.5  $\mu M$  (See Figure 25).

5        Example 10- Structure-Function Analysis of the CD36 Binding Domain of PfEMP1

To locate the minimal fragment of rC1-2 that binds CD36, and to identify the region in the rC1-2 RP which is important for binding, deletion mutants of RP rC1-2 were generated as described in  
10 Example 1(N), and their interaction with CD36 was tested. Deletion mutants were generated by PCR techniques known in the art, and as described herein. The mutants were immobilized on MAb 141.4 coated protein G sepharose beads, directly from the bacterial lysate. The binding of CD36 to the immobilized RP was tested with the RCRP assay  
15 (See Figure 19, and summarized in Table 5). Of those fragments tested, the smallest to retain the ability to bind CD36 was the 1-179 fragment. The 1-140 fragment did not bind to CD36, indicating that features important to binding of CD36 may lie within the segment including amino acids 140-179. Additionally, mutants 25-  
20 179, 25-192 or 11-179 did not bind CD36. The first 30 amino acids of rC1-2 are also expressed in rC1-1 which did not bind CD36. Thus, amino acids 1-10, 1-25 and 140-179 appear to be important, in combination, for CD36 binding. The possibility of other important regions located between these regions cannot be excluded. SDS-PAGE  
25 of rC1-2 under reduced and nonreduced conditions show a shift in mobility of the RP, indicating the possible existence of at least one disulfide bond. Replacement of cysteines 159 and 168 by serine, either together or separately, resulted in a reduction in the ability of the 1-179 fragment to bind CD36. However, all three  
30 separate mutations (cys-159-ser, cys-168-ser, and cys-159-ser in combination with cys-168-ser) retained some ability to bind CD36. These results are shown in Table 4, below.

In addition, each of the 5 cysteines at the 5' region of rC1-2 [1-179], CRM1 region, was replaced with a serine as described  
35 before. The binding of CD36 to the corresponding fusion protein is also described in Table 4.

Table 4

Binding of CD36 to Immobilized Fragments of rC1-2

5	rC1-2 Fragment	Binding of CD36
	rC1[1-233]	+++
	rC1[1-192]	+++
	rC1[1-179]	+++
	rC1[1-140]	-
10	rC1[1-102]	-
	rC1[1-87]	-
	rC1[1-59]	-
	rC1[1-30]	-
	rC1[25-233]	-
15	rC1[25-192]	-
	rC1[53-192]	-
	rC1[80-192]	-
	rC1[96-192]	-
	rC1[135-192]	-
20	rC1[156-192]	-
	rC1[156-233]	-
	rC1[1-179] ser 159	-/+
	rC1[1-179] ser 168	+
	rC1[1-179] ser 159 & 168	-/+
25	rC1-2 [1-179]	+++
	rC1-2[1-179] ser 45	-
	rC1-2[1-179] ser 49	++
	rC1-2[1-179] ser 32	N/A
	rC1-2[1-179] ser 41	N/A
30	rC1-2[1-179] ser 51	N/A
	rC1-2[1-179] reduced	+++
	rC1-2[1-179] red./alkyl.	-
	rC1-2[11-179]	-

35 N/A=Low expression of RP.

Reduction of rC1-2, followed by alkylation with iodoacetamide was associated with lower mobility on SDS-PAGE and no binding of CD36 (Table 4). Without alkylation, the protein refolded and bound CD36 (Table 4). Thus, binding of rC1-2 to CD36 appears to require a defined shape and is not entirely promoted by a simple linear sequence.

Example 11- Regions Homologous to rC1-2 Amplified from Other *P. falciparum* Strains

Using the primers derived from different parts of rC1-2, PCR products were generated from different *P. falciparum* strains. Primers corresponding to amino acids 1-233 and 1-179 as well as the universal primers gave PCR products from *P. falciparum* DNA only and failed to produce a product from the DNA of *P. cynemolgi*, *P. fragile*, *P. caotnyi* or *P. knowlesi*. The 1-233 fragment (complete rC1-2) was amplified only from MC strains of *P. falciparum* (K+C+R+, K+C+R-, K-C-R-), and not from the other strains tested. The 1-179 fragment gave PCR products from ten of eleven strains tested, and only failed to react with the Dd2 strain. Identical results were obtained when the fragments corresponding to 53-179, 53-140, 1-140 and 1-59 fragments were amplified. These strains and clones have different knob and adherence properties and express size and antigenically diversified PfEMP1 molecules. The PCR products amplified from the different strains appeared to be of similar size. That PCR product was generated among a variety of strains using primers corresponding to fragments 1-59 and 140-179 as well as the universal primers, indicates conservation of the sequences in these regions and also the apparent importance of these regions in ligand binding. The universal primers similarly gave products from gDNA and cDNA, first strand (RT-PCR) of all strains tested.

The binding of CD36 to immobilized GST-fusion proteins derived from the different *P. falciparum* strains is summarized in Table 5.

The alignment of the sequences obtained with the 1-179 primers from gDNA and from expressed vars (cDNA and RT-PCR) shows the 5'-end of the molecule (residues 1-78) and the 3'-end of the molecule (residues 140-180) are the more conserved part of the molecule (Figure 20). This conclusion is supported by the alignment

of the sequences obtained with the universal primers (Figures 20 and 21). Within these regions there are many residues that are identical in substantially all strains. These sequences are indicated as single amino acids in the consensus sequence shown in  
5 Figures 20 and 21. Some sequences show limited substitution of conserved sequences. These amino acid sequences are also indicated on the consensus alignment sequence. Accordingly, it appears that conserved, highly conserved and semi-conserved amino acids are important for the structure and function of CD36 binding domain,  
10 and can be used to model additional sequences with similar binding potential. The region of residues 80-140 is more degenerate and shows much less conservation. From this region, three types of sequences are apparent (See Figure 20). The first is the MC type including the three MC sequences MC R+ (cDNA and gDNA), MC R- and MC  
15 K-. The second is the "ItG" type that includes the gDNA sequences of strains FCR3-C5, FCR3-C6, ItG2-F6. ItG2-G1, ItG2-ICAM and Palo Alto and the cDNA sequence of FVO. The third group, named "HB3" contains the gDNA sequence of HB3 and the cDNA sequences of FCR-C5 and ItG2-F6. The binding of CD36 to immobilized rC1-2[1-179]  
20 polypeptides derived from the gDNA sequence of several different strains of *P. falciparum* is shown in Table 5. The alignment of these sequences was used to identify and prepare conserved degenerate universal oligonucleotides to PCR and identify different sequences corresponding to rC1-2 [10-151] from all *P. falciparum*  
25 strains and isolates.

With the universal primer sets unil79-5' and unil79-3', 12 out of 12 strains tested (MC R+, MC K-, MC R-, Dd2, FCR3-C5, FCR3-C6, HB3, ItG2-F6, ItG2-G1, It-ICAM, Palo Alto and cDNA of FVO and MC R+) produced a PCR product of similar size. The PCR results  
30 indicated that more than one product was produced in many preparations. Some of the obtained sequences were almost identical or very similar to the sequence obtained with the 1-179 primer set. However, other clones differed from those obtained with the 1-179 primers. The sequence of the Dd2 strain was almost identical to the  
35 published sequence of the Dd2 var-7 gene (Su et al., supra). The cysteines of CRM-1, located at the 30-55 region of the alignment are conserved in all strains. However, some sequences show different spacing between the cysteines and are of the form of

CIN(D)X<sub>6-7</sub>CI(K)X<sub>2-4</sub>CX<sub>2</sub>K(D)CXCF. Additionally, conserved sequences are found from position 10 (FWXWVXXMLXDS"XWR(K) and the sequence in the region of residue 140 (i.e., TTIDK(X)LXH. Additional conserved amino acids are found at different locations of the alignment

- 5 (Figures 20 and 21). The above data demonstrate that the uni179 primer sets are very useful tools for obtaining and identifying that portion of the rCl-2 [1-179] region involved in adherence to CD36 from different strains of *P. falciparum*. These universal primers have been effectively used to amplify sequences from every strain
- 10 tested. The PCR products of the universal primers can also be used to identify and make fingerprints from strains, clones or isolates of *P. falciparum*. In particular, the PCR products originating from a particular sample can be labeled according to known labeling methods (e.g., those described herein) and separated on a sequencing
- 15 gel capable of separating large fragments (i.e., >500 bases) that only differ in size by one or two nucleotides. This method can also detect changes in the expressed var by RT-PCR similar to Smith et al. Some such changes may be related to phenotypic changes in the adherence properties of the PE.

20

Table 5

<i>P. falciparum</i> Strain	Binding to CD36
MC R+ (cDNA derived)	+++
MC R+	+++
25 MC R-	+++
MC K-	+++
ItG2-G1	+/-
ItG2-F6	+/-
ItG2-ICAM	+/-
30 FCR3-C5	+
FCR3-C6	+/-
HB3	+/-
PA	-

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FVO (cDNA derived)	-
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Fusion proteins derived from the MC strain showed strong binding to CD36. Recombinant proteins derived from sequences of other strains had little or no detectable binding to CD36. This was true for proteins derived from the expressed var gene of adherence positive strains (FVO, ItG2-F6 and FCR-C5) as well for recombinants derived from gDNA sequences. This results from inappropriate folding of the recombinant protein in the bacterial host, as the protein contains 7 cysteine residues.

Chimeric proteins were prepared which were composed in part from the sequence of the strong CD36 binding recombinant protein of the MC parasites complemented with a sequence from week or non-reactive rC1-2 [1-179] clones. Six chimeric proteins were prepared and tested for binding of CD36 (Table 6). One of these chimeric proteins MC R+/ItG2-F6 had substantial binding (about 50% of the binding of MC R+ rC1-2 [1-179]). Several other proteins including MC R+/HB3, MC R+/FCR3-C5 and FCR3-C5/MC R+ had lower binding activities. The positive binding of the chimeric MC R+/ItG2-F6 recombinant protein indicates that the rC1-2 [1-179] is involved with binding to CD36 and that the inability to obtain high-binding recombinant proteins from strains other than MC is most likely due to incorrect folding of these recombinant proteins in *E. coli*. This procedure is particularly important for sequences generated with the universal primers since these sequences lack the two 3' cysteine codons which are important for binding. The above chimeric protein clone, with a 5' portion from a universal primer and a 3' clone from one of the rC1-2[1-179] sequences will have the 3' cysteines important for its function.

Table 6

Chimeric Recombinant Protein (5' / 3' sequence)	Binding of CD36
HB3/MC	-

	ItG2-F6/MC	-
	FCR3-C5/MC	+/-
	MC /HB3	+/-
	MC/ItG2-F6	+++
5	MC/FCR3-C5	+

In an effort to overcome possible folding problems, expression was also tested in eukaryotic expression systems. rC1-2[1-179] ("MC") transiently expressed on the surface of COS-7 cells bound to CD36. The rC1-2[1-179]-ItG construct did not bind to CD36 while the HB3 construct showed very low level expression. The results of these expressions are shown in Table 7, below.

15

Table 7

Properties of rC1-2[1-179] Expressed in Eukaryotic Expression Systems

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25

30

Sequence	Expression System	Expression	CD36 Binding	Blockade of PE Adherence
rC1-2[1-179]MC	COS-7	++	++	N/A
" ItG	COS-7	++++	-	N/A
" HB3	COS-7	+	-	N/A
" MC	Yeast	+	-	-
" FVO	Yeast	+	++	++

cleaved product.

However, expression of the rC1-2[1-179]("FVO") sequence with a terminal six histidine tag in yeast produced a correctly folded protein that bound to CD36 and blocked 50% adherence ( $IC_{50}$ ) of MC PE to CD36 at 50  $\mu$ g/ml and gave 70% blockage at 100  $\mu$ g/ml. The  
5 rC1-2[1-179] "MC" product appeared to be proteolytically cleaved and did not bind CD36 or block adherence of PE. Thus, the recombinant rC1-2[1-179] region from two different *P. falciparum* parasites was shown to mediate adherence of PE to CD36.

10 VI. Summary of Results

PfEMP1 has been attributed the dual properties of antigenic variation on the surface of *P. falciparum* parasitized erythrocytes ("PE"), and receptor properties of adherence to host  
15 proteins on microvascular endothelial cells. For a review of these findings, see, e.g., Howard and Gilladoga, (1989), and Pasloske and Howard, (1994), *supra*. PfEMP1 is therefore at the crux of understanding the molecular pathogenesis of *P. falciparum* malaria insofar as it involves antigenic variation and evasion of  
20 antimalarial immunity, as well as PE sequestration and the consequent vascular obstruction. The molecular basis for these phenomena has languished however since repeated attempts to clone PfEMP1 have failed.

Several independent criteria establish that the genes described herein encode the PfEMP1 protein of K+ MC strain  
25 *P. falciparum*. The relevant results are summarized in Tables 8 and 9.



Table 8

Properties of antisera against PfEMP1 recombinant fusion proteins.<sup>a</sup>

5	Immunogen <sup>b</sup>	Species	PfEMP1 immuno-ppt <sup>c</sup>	PE aggl. <sup>d</sup>	PE IFA <sup>e</sup>	Blockade of PE adherence <sup>f</sup>
	rB1 (161-385)	rabbit	0/2	0/2	NDg	0/2
	rC1-2 (576-808)	rabbit	3/4	2/4 <sup>h</sup>	0/2	0/4
10	rC1-2 (576-808)	rat	4/4	4/4 <sup>i</sup>	4/4	4/4
	rD1 (818-1003)	rabbit	2/2	0/2	ND	0/2
15	rD1 (818-1003)	rat	ND	3/4 <sup>j</sup>	ND	0/2

a. Results shown as [number of antisera scoring positive for property]/[number of animals immunized].

20 b. GST fusion proteins corresponding to fragments of MC K+ PfEMP1.

c. Immunoprecipitation of <sup>125</sup>I-PfEMP1 from SDS-extract of MC K+ PE.

d. Agglutination at 1:5 dilution of intact mature stage PE of MC K+ PE.

25 e. Indirect immunofluorescence with intact non-fixed MC K+ PE.

f. Blockade of adherence of MC K+ PE to immobilized CD36 at 1:5 serum dilution.

g. ND: not determined.

30 h. Agglutination was detected after disruption of rosettes.

i. Agglutination was observed with sera of day >195 of PE from strains MC, ItG-ICAM and ItG2-F6.

j. Agglutination was observed after disruption of rosettes.

35 Agglutination was observed for parasitized erythrocytes of MC R+ and ItG-ICAM and ItG2-F6 strains.

Rat sera tested, were found to be active from second immunization and with monthly boosters, were also reactive beyond day 200.

40

Table 9

Properties of antisera against PfEMP1 recombinant fusion proteins

5

	Immunogen	Fusion	Species	PfEMP1 Immuno ppt	Tryp. Frag. Immunoppt	Block PE adher	PE aggl
	rA1[3-158]	GST	rat	0/4			0/4
	rA1[3-158]	MBP	rat				0/3
	rB1[161-385]	GST	rat	1/4			0/8
10	rC1-1[402-605]	MBP	rat	0/4	0/1		1/4
	rC1-2[576-808]	GST	rabbit	2/2		0/2	2/2
	rC1-2[576-808]	GST	goat			1/2	2/2
	rC1-2[576-808]	GST	rat	4/4	4/4 (TF90)	4/4	8/8
	rC1-2[1-179]-MC	GST	rat	8/8	4/4 (TF90)	4/8	8/8
15	rC1-2[1-179]-MC	MBP	rat				3/4
	rC1-2[1-179]- "ItG"	GST	rat				0/4
	rC1-2[1-179]- "ItG"	MBP	rat				0/4
20	rC1-2[1-179]- "HB3"	MBP	rat				0/2
	rD1[818-1003]	GST	rat	4/4	4/4 (TF125)	0/4	4/4
	rD1[808-1003]	GST	rabbit	2/2		0/2	0/2
	rD2[982-1320]	GST	rat	0/4			0/4
25	rF1-1[1300-1707]	GST	rat	8/8	4/4 (TF125)		7/8
	rF1-3[2171-2450]	GST	rat	3/4	2/2 (TF125)		2/4
	rG1[2550-2794]	GST	rat	4/4	0/1		0/4
	rD3[992-1243]	GST	rat				0/4
	rE1-1[1219-1471]	GST	rat	0/4	0/2		1/4
30	rE1-2[1454-1719]	GST	rat	0/4	0/2		1/4

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Table 10

Properties of antisera against PfEMP1 recombinant fusion proteins

5

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25

30

35

40

Immunogen	Fusion	Species	MC R+	ItG-ICAM	PA
rA1[3-158]	GST	rat	0/4		
rA1[3-158]	MBP	rat	0/3		
rB1[161-385]	GST	rat	0/8		
rC1-1[402-605]	MBP	rat	1/4		
rC1-2[576-808]	GST	rabbit	2/2		
rC1-2[576-808]	GST	goat	2/2	0/2	0/2
rC1-2[576-808]	GST	rat	8/8	5/6	6/6
rC1-2[1-179]-MC	GST	rat	8/8	8/8	
rC1-2[1-179]-MC	MBP	rat	3/4	0/2	2/2
rC1-2[1-179]-"ItG"	GST	rat	0/4	1/4	1/2
rC1-2[1-179]-"ItG"	MBP	rat	0/4	2/4	1/2
rC1-2[1-179]-"HB3"	MBP	rat	0/2	1/2	2/2
rD1[818-1003]	GST	rat	4/4	4/4	0/4
rD2[982-1320]	GST	rat	0/4		
rF1-1[1300-1707]	GST	rat	7/8		
rF1-3[2171-2450]	GST	rat	2/4		
rG1[2550-2794]	GST	rat	0/4		
rD3[992-1243]	GST	rat	0/4		

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	rE1- 1(1219- 1471]	GST	rat	1/4		
5	rE1- 2[1454- 1719]	GST	rat	1/4		

Some sera tested did not agglutinate PE from strains MC R-, ItG2-G1, FCR3-C5, Dd2, D10 and HB3. PE from strain ItG2-F6 were agglutinated by normal rat sera and thus could not be tested.

PfEMP1, as identified by immunoprecipitation of  $^{125}\text{I}$ -labeled PE surface proteins, has been shown to be antigenically diverse with different parasite strains and clones (Leech et al., *J. Exp. Med.* (1984) 159:1567-1575); Howard et al., (1988), *supra*; Schravendijk et al., (1991), *supra*; Biggs et al., *J. Immunol.* (1992) 149:2047-2054). Antibodies generated in multiple animals by immunization with RP derived from two different parts of the cDNA immunoprecipitate  $^{125}\text{I}$ -PfEMP1 only from MC strain PE and failed to immunoprecipitate PfEMP1 from the PfEMP1 bearing PE of strain ItG2-ICAM. The  $^{125}\text{I}$ -immunoprecipitated protein was defined as PfEMP1 by its molecular size, specific detergent extraction properties and sensitivity to low levels of trypsin (Aley et al., (1984); Leech et al., *J. Exp. Med.* (1984) 159:1567-1575; Howard et al., (1988), *supra*). Competition experiments with the two recombinant proteins ("RP") proved that the capacity of these anti-recombinant sera to immunoprecipitate  $^{125}\text{I}$ -PfEMP1 was not due to the presence of a cross-reactive epitopes on these immunogens. Western blotting with different strains and clones indicated that the anti rC1-2 serum was reactive with variable size bands, including some which show migration similar to  $^{125}\text{I}$ -PfEMP1 from these strains (Howard et al., (1988), *supra*; van Schravendijk et al., (1991), *supra*). However, much more cross-reactivity is apparent among fully unfolded PfEMP1s, as with Western blotting, than among the native proteins.

Second, since PfEMP1 is expressed as a variant protein on the PE surface (Howard et al., *Molec. Biochem. Parasitol.* (1988) 27:207-223; van Schravendijk et al., (1991), *supra*; Roberts et al., *Nature* (1992) 318:64-66), sera raised against the RP should react in a strain-specific manner with the surface of intact PE. Rat anti-rC21-2 sera reacted with the surface of intact MC K+ PE by

mediating specific agglutination, by indirect immunofluorescence and by immunoelectronmicroscopy. This reactivity was specific to MC K+ strain and was not evident with any other strain including the MC K- strain known to lack surface-exposed PfEMP1 (Aley et al., (1984)).

5 However, sera raised to rD1 showed cross-reactive agglutination of PE from several strains (MC R+, ItG2-ICAM and ItG2-F6) but not other strains (MC R-, ItG2-G1, Palo Alto, FCR3-C5 and Dd2). The same was observed with rat serum to rC1-2 of day >195. Agglutination of PE from several strains (but not all) by rat serum to rD1 or serum to  
10 rC1-2 of day >195 suggests the expression of cross-reactive epitopes of PfEMP1 on the PE surface and that these epitopes normally do not elicit antibodies during natural course of infection in monkeys or humans. These cross-reactive epitopes may be limited to a subset of PfEMP1 variants. Human sera from an adult subject living in an  
15 endemic area, eluted from the surface of PE, has been shown to agglutinate PE of several strains (Marsh and Howard, *Science* (1986), *supra*), which raises the possibility that cross-reactive epitopes can be expressed on the surface of PE of different isolates. The surface accessibility of these and other epitopes of PfEMP1 can be  
20 greatly reduced by the formation of rosettes, as demonstrated by the increase in PE agglutination by anti-PfEMP1 sera, upon disruption of rosettes. Thus, formation of rosettes may confer an advantage to the parasite by reducing the accessibility of antibodies to the surface of the PE.

25 Reactivity of these anti-PfEMP1 antibodies on immunoelectronmicroscopy was restricted to the knob protrusions on PE, in agreement with earlier studies that demonstrated specific binding of isolate-specific antibodies to knobs (van Schravendijk et al., (1991), *supra*, and specific binding of CD36 and TSP to knobs  
30 (Nakamura et al., *J. Histochem. Cytochem.* (1992) 40:1419-1422).

Finally, PfEMP1 has been associated with the property of adherence of PE to CD36 and other endothelial cell surface proteins (Howard and Gilladoga, (1989), *supra*). Sera raised against rC1-2 specifically blocked PE adherence to CD36. The ability of sera to  
35 block adherence of PE was generally correlated with agglutination of the same PE (Howard et al., (1988), *supra*; Iqbal et al., *Trans. R. Soc. Trop. Med. Hyg.* (1993) 87:583-588). The results obtained with the anti rC1-2 sera support and verify these observations.

Furthermore, the RP, rC1-2 binds to CD36 and blocks and reverses the adherence of several strains to CD36. These results prove that PE binding to CD36 is mediated by PfEMP1.

Analysis of the MC PfEMP1 sequence would predict that each  
5 cDNA clone would hybridize to a single band of Eco RI or Eco RI/Hind  
III digested DNA. In contrast, hybridization to more than one band  
was apparent with every clone, indicating more than one gene copy in  
the parasite genome. This is supported by the data showing two  
variants of the MC strain PfEMP1 gene. Thus, *P. falciparum*  
10 parasites have a family of PfEMP1 genes that constitute shared and  
diverse sequences. PfEMP1 displays antigenic variation with  
extremely high frequency (Roberts et al., (1992), *supra*). Antigenic  
switching is apparently associated with expression of a different  
PfEMP1 gene. Thus, antigenic differences in the PfEMP1  
15 extracellular domains, a property expected from the earlier serology  
(Marsh and Howard, *Science* (1986) 231:150-153; Aguiar et al.,  
(1992), *supra*; Iqbal et al., (1993), *supra*) and immunochemical  
studies (Leech et al., *J. Exp. Med.* (1984) 159:1567-1575; Howard et  
al., (1988), *supra*), derive from these sequence differences.  
20 Within the protein, large domains can be identified with  
clear homology to the Duffy Binding Ligand domains characteristic of  
EBA-175 and DABP. The MC PfEMP1 sequence possesses four of these  
domains, defined by particular sequence motifs. In view of the  
known property of the DBL domains to participate in the binding of  
25 merozoite surface proteins to erythrocyte proteins (Chitnis and  
Miller, *J. Exp. Med.* (1994) 180:497-506), it might be anticipated  
that these domains represent the regions responsible for adherence  
of PfEMP1 to CD36, TSP and other proteins expressed on endothelial  
cells. The rC1-2 RP which elicited adherence-blocking antibodies  
30 lacked any portion of the DBL domains but included the CRM-1  
cysteine-rich motifs. The cysteines in this CRM-1 motif, CX<sub>3</sub>CX<sub>3</sub>CXC  
are conserved among different strains. Although the amino acid  
motif CX<sub>3</sub>CX<sub>3</sub>CXC occurs in only a small number of animal proteins,  
including human von Willebrand's Factor as well as in numerous plant  
35 protein sequences, it is not identified with any specific structural  
or biologic function. Also of potential relevance to the adherence  
properties of PfEMP1 was the observation of an RGD motif and an LDV  
motif, both associate with protein-protein interaction and cells

attachment (for review, Kuhn and Eble, Trends Cell Biol. (1994) 4:256-261). These motifs occur in some PfEMP1 sequences but not in others. One of the MC K+ PfEMP1 sequences includes a RGD motif, while the PfEMP1 variant sequence represented by the D3-E1-cDNA  
5 lacked this motif. The appearance of such motifs in only some of the PfEMP1 genes may explain the extraordinary diversity and plasticity of PE adherence phenotype.

Antibodies to rC1-2 block PE adherence to CD36 but not to TSP. In separate studies it has been demonstrated that different  
10 tryptic fragments of <sup>125</sup>I-PfEMP1 released from the surface of MC K+ PE bind to TSP and CD36, suggesting that these receptor properties reside in different parts of the PfEMP1 protein. The capacity of anti-rC1-2 serum antibodies to immunoprecipitate the same tryptic fragment affinity purified by CD36 and not with TSP and to block and  
15 reverse adherence to CD36 but without effect on adherence to TSP is consistent with these observations. rC1-2, encoded by clone C1, specifically bound to CD36, and not to other host cell receptors, including TSP and ICAM-1. This RP also blocked the adherence of PE to CD36, but did not effect binding to TSP. In contrast to the  
20 strain specific blockade of adherence shown by rC1-2 sera and other blocking anti-PE sera, rC1-2 itself, blocked adherence of PE from four separate strains to CD36, indicating that different *P. falciparum* strains bind to the same region (139-155) of CD36. Structure function analysis of rC1-2 reveals a binding fragment  
25 consisting of amino acids 1-179. Within this fragment, the first 25 amino acids appear to be important for binding. The cysteines at positions 159-168 also appear to be important for binding, indicating an important tertiary structure. The CRM-1 region, conserved in several strains, as well as the region defined by  
30 residues 140-179 also appear to have some role in the structure or binding activity. Conserved residues among different var genes (figs. 20 and 21) appear to be important to the structure and function of the CD36 binding domain of *P. falciparum* parasites

Solubilized PfEMP1 has also been found to bind CD-36, TSP  
35 or ICAM-1. Similarly, tryptic fragments cleaved from PE surface bind to CD36 and TSP. This and other data, above, confirms that PfEMP1 is responsible for both the antigenic variation and receptor

properties on PE, which are central to the special virulence and pathology of *P. falciparum*.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.



WHAT IS CLAIMED IS:

1                   1. A substantially pure polypeptide, comprising a PfEMP1  
2 protein or a biologically active fragment or analogue thereof.

1                   2. The polypeptide of claim 1, wherein the polypeptide  
2 comprises an amino acid sequence which is substantially homologous  
3 to an amino acid sequence shown in Figure 2, a biologically active  
4 fragment or analogue thereof.

1                   3. The polypeptide of claim 2, wherein the polypeptide  
2 comprises an amino acid sequence which is substantially homologous  
3 to amino acids 576 through 755 of the amino acid sequence shown in  
4 Figure 2.

1                   4. The polypeptide of in claim 3, wherein the  
2 polypeptide comprises an amino acid sequence which is substantially  
3 homologous to amino acids 576 through 808 of the amino acid sequence  
4 shown in Figure 2.

1                   5. The polypeptide of claim 1, wherein the polypeptide  
2 further comprises a heterologous protein fused to said amino acid  
3 sequence substantially homologous to the amino acid sequence of  
4 PfEMP1, or biologically active fragment thereof.

1                   6. A substantially pure polypeptide, said polypeptide  
2 comprising an amino acid sequence encoded by a nucleic acid sequence  
3 that is capable of hybridizing with at least one oligonucleotide in  
4 a mixture of oligonucleotides wherein each oligonucleotide in said  
5 mixture comprises a general nucleotide sequence:

6                   ACX<sub>6</sub>ACA ATT GAT AAA X<sub>7</sub>TX<sub>8</sub> CTX<sub>9</sub> X<sub>10</sub>AX<sub>11</sub> CAC GAA

7 where X<sub>6</sub> is selected from C and T, X<sub>7</sub> is selected from T and A, X<sub>8</sub> is  
8 selected from G and A, X<sub>9</sub> is selected from C and A, X<sub>10</sub> is selected  
9 from C and A and X<sub>11</sub> is selected from T and A.

1           7. A substantially pure polypeptide, said polypeptide  
2 comprising an amino acid sequence that may be encoded by a nucleic  
3 acid sequence that is capable of hybridizing with at least one  
4 oligonucleotide in a mixture of oligonucleotides wherein each  
5 oligonucleotide in said mixture comprises the general nucleotide  
6 sequence:

7           TTT TTT TGG X<sub>12</sub>X<sub>13</sub>X<sub>14</sub> TGG GTX<sub>15</sub> X<sub>16</sub>X<sub>17</sub>X<sub>18</sub> X<sub>19</sub>AX<sub>20</sub> ATG TTA

8           where X<sub>12</sub> is selected from G and A, X<sub>13</sub> is selected from A  
9 and T, X<sub>14</sub> is selected from G and T, X<sub>15</sub> is selected from A and T,  
10 X<sub>16</sub> is selected from T, A and C, X<sub>17</sub> is selected from A and C, X<sub>18</sub>  
11 is selected from T and C, X<sub>19</sub> is selected from G and C and X<sub>20</sub> is  
12 selected from T and A.

1           8. A substantially pure polypeptide, said polypeptide  
2 comprising one or more of general amino acid sequences:

3  
4           TTIDKX<sub>1</sub>LX<sub>2</sub>HE; and  
5           FFWX<sub>3</sub>X<sub>4</sub>VX<sub>5</sub>X<sub>6</sub>ML

6           where X<sub>1</sub> is selected from leucine or isoleucine, X<sub>2</sub> is  
7 selected from glutamine and asparagine, X<sub>3</sub> is selected from  
8 methionine, lysine and aspartic acid, X<sub>5</sub> is selected from histidine,  
9 threonine and tyrosine and X<sub>6</sub> is selected from aspartic acid,  
10 glutamic acid and histidine.

1           9. The polypeptide of claim 8, said polypeptide  
2 comprising an amino acid sequence substantially homologous to an  
3 amino acid sequences selected from the group consisting of an amino  
4 acid sequence of r179 MC K+R+, r179 MC K-, r179 MC R-, r179-C5,  
5 r179-C6, r179-ItG F6, r179-ItG 2G1, r179-ItG ICAM, r179 PA and r179-  
6 HB3 as shown in Figure 20.

1           10. An isolated nucleic acid, said nucleic acid  
2 comprising at least about 15 contiguous nucleotides and encoding a  
3 PfEMP1 protein or a biologically active fragment thereof.

1           11. The isolated nucleic acid of claim 10, wherein the  
2 nucleic acid encodes an amino acid sequence which is substantially  
3 homologous to amino acids 576 through 755 of the amino acid sequence  
4 shown in Figure 2.

1           12. The isolated nucleic acid of claim 10, wherein the  
2 nucleic acid encodes an amino acid sequence which is substantially  
3 homologous to amino acids 576 through 808 of the amino acid sequence  
4 shown in Figure 2.

1           13. The isolated nucleic acid of claim 10, wherein the  
2 nucleic acid comprises at least 15 contiguous nucleotides from the  
3 nucleotide sequence shown in Figure 12.

1           14. The isolated nucleic acid of claim 10, wherein the  
2 nucleic acid comprises at least 50 contiguous nucleotides from the  
3 nucleotide sequence shown in Figure 12.

1           15. The isolated nucleic acid as recited in claim 10,  
2 wherein the nucleic acid comprises a nucleotide sequence which is  
3 substantially homologous to the nucleotide sequence shown in Figure  
4 12.

1           16. An isolated nucleic acid, said nucleic acid  
2 comprising at least about 15 contiguous nucleotides and being  
3 capable of hybridizing to at least one oligonucleotide from a  
4 mixture of oligonucleotides wherein each oligonucleotide in said  
5 mixture comprises a general nucleotide sequence:

6                   ACX<sub>6</sub>ACA ATT GAT AAA X<sub>7</sub>TX<sub>8</sub> CTX<sub>9</sub> X<sub>10</sub>AX<sub>11</sub> CAC GAA

7       where X<sub>6</sub> is selected from C and T, X<sub>7</sub> is selected from T and A, X<sub>8</sub> is  
8 selected from G and A, X<sub>9</sub> is selected from C and A, X<sub>10</sub> is selected  
9 from C and A and X<sub>11</sub> is selected from T and A.

1           17. An isolated nucleic acid, said nucleic acid  
2 comprising at least about 15 contiguous nucleotides and being  
3 capable of hybridizing to at least one oligonucleotide from a

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4 mixture of oligonucleotides wherein each oligonucleotid in said  
5 mixture comprises a general nucleotide sequence:

6 TTT TTT TGG  $X_{12}X_{13}X_{14}$  TGG GTX<sub>15</sub>  $X_{16}X_{17}X_{18}$   $X_{19}AX_{20}$  ATG TTA

7 where  $X_{12}$  is selected from G and A,  $X_{13}$  is selected from A and T,  $X_{14}$   
8 is selected from G and T,  $X_{15}$  is selected from A and T,  $X_{16}$  is  
9 selected from of T, A and C,  $X_{17}$  is selected from A and C,  $X_{18}$  is  
10 selected from of T and C,  $X_{19}$  is selected from G and C and  $X_{20}$  is  
11 selected from T and A.

1 18. The nucleic acid of claim 17, wherein said nucleic  
2 acid encodes a polypeptide comprising an amino acid sequence  
3 selected from the group consisting of an amino acid sequence of r179  
4 MC K+R+, r179 MC K-, r179 MC R-, r179-C5, r179-C6, r179-ItG F6,  
5 r179-ItG 2G1, r179-ItG ICAM, r179 PA and r179-HB3 as shown in Figure  
6 20.

1 19. A nucleic acid probe, said probe comprising a general  
2 nucleotide sequence:

3 ACX<sub>6</sub>ACA ATT GAT AAA  $X_7TX_8$  CTX<sub>9</sub>  $X_{10}AX_{11}$  CAC GAA

4 where  $X_6$  is selected from C and T,  $X_7$  is selected from T and A,  $X_8$  is  
5 selected from G and A,  $X_9$  is selected from C and A,  $X_{10}$  is selected  
6 from C and A and  $X_{11}$  is selected from T and A.

1 20. The nucleic acid probe of claim 19, wherein said  
2 probe is capable of hybridizing with a nucleic acid which encodes a  
3 PfEMP1 polypeptide or biologically active fragment thereof.

1 21. A nucleic acid probe, said probe comprising a general  
2 nucleotide sequence:

3 TTT TTT TGG  $X_{12}X_{13}X_{14}$  TGG GTX<sub>15</sub>  $X_{16}X_{17}X_{18}$   $X_{19}AX_{20}$  ATG TTA

4 where  $X_{12}$  is selected from G and A,  $X_{13}$  is selected from A and T,  $X_{14}$   
5 is selected from G and T,  $X_{15}$  is selected from A and T,  $X_{16}$  is

6 selected from of T, A and C, X<sub>17</sub> is selected from A and C, X<sub>18</sub> is  
7 selected from of T and C, X<sub>19</sub> is selected from G and C and X<sub>20</sub> is  
8 selected from T and A.

1           22. The nucleic acid probe of claim 21, wherein said  
2 probe is capable of hybridizing with a nucleic acid which encodes a  
3 PfEMP1 polypeptide or biologically active fragment thereof.

4           23. An expression vector, said expression vector  
5 comprising a nucleic acid segment operably linked to a promoter  
6 sequence, wherein said nucleic acid segment encodes a PfEMP1 protein  
7 or biologically active fragment thereof.

1           24. A method of preparing a PfEMP1 polypeptide or  
2 biologically active fragment thereof comprising:  
3                 inserting into an expression vector a nucleic acid  
4 which encodes a PfEMP1 polypeptide or biologically active fragment  
5 thereof;  
6                 transfecting a host cell capable of expressing said  
7 nucleic acid with said expression vector to express said PfEMP1  
8 polypeptide or biologically active fragment; and  
9                 recovering said expressed PfEMP1 polypeptide or  
10 biologically active fragment.

1           25. A recombinant host cell, wherein said host cell has  
2 been transfected with an expression vector comprising a nucleic acid  
3 segment operably linked to a promoter sequence, wherein said nucleic  
4 acid segment encodes a PfEMP1 protein or biologically active  
5 fragment thereof, whereby said cell is capable of expressing said  
6 nucleic acid.

7           26. The recombinant host cell of claim 25, wherein said  
8 host cell is a CHO cell.

1           27. An isolated antibody, wherein said antibody is  
2 specifically immunoreactive with a PfEMP1 polypeptide or a  
3 biologically active fragment thereof.

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1           28. The antibody of claim 27, wherein said antibody is  
2 specifically immunoreactive with a polypeptide which comprises an  
3 amino acid sequence substantially homologous to an amino acid  
4 sequence shown in Figure 2, or a biologically active fragment  
5 thereof.

1           29. The antibody of claim 27, wherein said antibody is  
2 specifically immunoreactive with a polypeptide which comprises an  
3 amino acid sequence substantially homologous to amino acids 576  
4 through 755 of the amino acid sequence shown in Figure 2.

1           30. The antibody of claim 27, wherein said antibody is  
2 specifically immunoreactive with a polypeptide which comprises an  
3 amino acid sequence substantially homologous to amino acids 576  
4 through 808 of the amino acid sequence shown in Figure 2.

1           31. The antibody of claim 27, wherein said antibody is  
2 specifically immunoreactive with a polypeptide comprising one or  
3 more of general amino acid sequences:

4  
5                   TTIDKX<sub>1</sub>LX<sub>2</sub>HE; and  
6                   FFWX<sub>3</sub>X<sub>4</sub>VX<sub>5</sub>X<sub>6</sub>ML

7           where X<sub>1</sub> is selected from leucine or isoleucine, X<sub>2</sub> is  
8 selected from glutamine and asparagine, X<sub>3</sub> is selected from  
9 methionine, lysine and aspartic acid, X<sub>5</sub> is selected from histidine,  
10 threonine and tyrosine and X<sub>6</sub> is selected from aspartic acid,  
11 glutamic acid and histidine.

1           32. The antibody of claim 29, wherein said antibody is  
2 specifically immunoreactive with a polypeptide having an amino acid  
3 sequence selected from the group consisting of an amino acid  
4 sequence of r179 MC K+R+, r179 MC K-, r179 MC R-, r179-C5, r179-C6,  
5 r179-ItG F6, r179-ItG 2G1, r179-ItG ICAM, r179 PA and r179-HB3 as  
6 shown in Figure 13.

1           33. A pharmaceutical composition, comprising the  
2 polypeptide of claim 1, in a pharmaceutically acceptable carrier.

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1           34. A pharmaceutical composition, comprising the nucleic  
2 acid of claim 10, in a pharmaceutically acceptable carrier.

1           35. A pharmaceutical composition comprising the antibody  
2 of claim 27, in a pharmaceutically acceptable carrier.

1           36. A method of inhibiting an interaction between PfEMP1  
2 and a PfEMP1-ligand, comprising contacting the PfEMP1-ligand with an  
3 effective amount of a PfEMP1 polypeptide or biologically active  
4 fragment thereof.

1           37. A method of inhibiting an interaction between a  
2 PfEMP1 polypeptide and a PfEMP1-ligand, comprising contacting the  
3 PfEMP1 polypeptide with an effective amount of an antibody that is  
4 specifically immunoreactive with a PfEMP1 polypeptide or  
5 biologically active fragment thereof.

1           38. The method of claim 37, wherein the PfEMP1 ligand is  
2 selected from the group consisting of CD36, VCAM, ELAM, ICAM and  
3 TSP.

1           39. A method of treating a patient suffering from  
2 symptoms of a malaria parasite infection, comprising administering  
3 to the patient, an effective amount of the pharmaceutical  
4 composition of claim 33.

1           40. The method of claim 39, wherein the symptom of  
2 malaria parasite infection is sequestration of erythrocytes.

1           41. The method of claim 39, wherein the malaria parasite  
2 is *P. falciparum*.

1           42. A method of treating a patient suffering from  
2 symptoms of a malaria parasite infection, comprising administering  
3 to the patient, an effective amount of the pharmaceutical  
4 composition of claim 34.

1           43. The method of claim 42, wherein the symptom of  
2 malarial infection is sequestration of erythrocytes.

1           44. The method of claim 42, wherein the malaria parasite  
2 is *P. falciparum*.

1           45. A method of preventing symptoms of a malaria parasite  
2 infection in a patient, comprising administering to the patient an  
3 effective amount of the pharmaceutical composition of claim 33.

1           46. The method of claim 45, wherein the malaria parasite  
2 is *P. falciparum*.

1           47. The method of claim 45, wherein said administering  
2 step is prior to the malaria parasite infection, and the effective  
3 amount is an immunogenically effective amount.

1           48. A method of preventing symptoms of a malaria parasite  
2 infection in a patient, comprising administering to the patient an  
3 effective amount of the pharmaceutical composition of claim 35.

1           49. The method of claim 48, wherein the malaria parasite  
2 is *P. falciparum*.

1           50. The method of claim 48, wherein said administering  
2 step is prior to the malaria parasite infection, and the effective  
3 amount is an immunogenically effective amount.

1           51. A method of determining whether a test compound is an  
2 antagonist of PfEMP1/PfEMP1-ligand complex formation, comprising the  
3 steps of:

4           incubating the test compound with a PfEMP1 polypeptide, or  
5 a biologically active fragment thereof, and the PfEMP1-l  
6 the PfEMP1-ligand, under conditions which permit the formation of  
7 the complex;

8           determining the amount of complex formed during said  
9 incubating step, and comparing that amount with the amount of  
10 complex formed in the absence of the test compound, a decrease in



11 the amount of the complex formed in the presence of the test  
12 compound being indicative that the compound is an antagonist of  
13 PfEMP1/PfEMP1-ligand complex formation.

1           52. The method as recited in claim 51, wherein the  
2 PfEMP1-ligand is CD36.

1           53. A method of identifying a *P. falciparum* parasite,  
2 comprising:  
3           amplifying nucleic acids from said parasite using a 3'  
4 primer sequence having a general nucleotide sequence:

5           ACX<sub>6</sub>ACA ATT GAT AAA X<sub>7</sub>TX<sub>8</sub> CTX<sub>9</sub> X<sub>10</sub>AX<sub>11</sub> CAC GAA

6 where X<sub>6</sub> is selected from C and T, X<sub>7</sub> is selected from T and A, X<sub>8</sub> is  
7 selected from G and A, X<sub>9</sub> is selected from C and A, X<sub>10</sub> is selected  
8 from C and A and X<sub>11</sub> is selected from T and A, and a 5' primer  
9 sequence having a general nucleotide sequence:

10           TTT TTT TGG X<sub>12</sub>X<sub>13</sub>X<sub>14</sub> TGG GTX<sub>15</sub> X<sub>16</sub>X<sub>17</sub>X<sub>18</sub> X<sub>19</sub>AX<sub>20</sub> ATG TTA

11 where X<sub>12</sub> is selected from G and A, X<sub>13</sub> is selected from A and T, X<sub>14</sub>  
12 is selected from G and T, X<sub>15</sub> is selected from A and T, X<sub>16</sub> is  
13 selected from of T, A and C, X<sub>17</sub> is selected from A and C, X<sub>18</sub> is  
14 selected from of T and C, X<sub>19</sub> is selected from G and C and X<sub>20</sub> is  
15 selected from T and A;

16           generating a characteristic pattern of said amplified  
17 nucleic acids; and

18           comparing said characteristic pattern of said amplified  
19 nucleic acids to a known characteristic pattern of amplified nucleic  
20 acids from a known *P. falciparum* strain.

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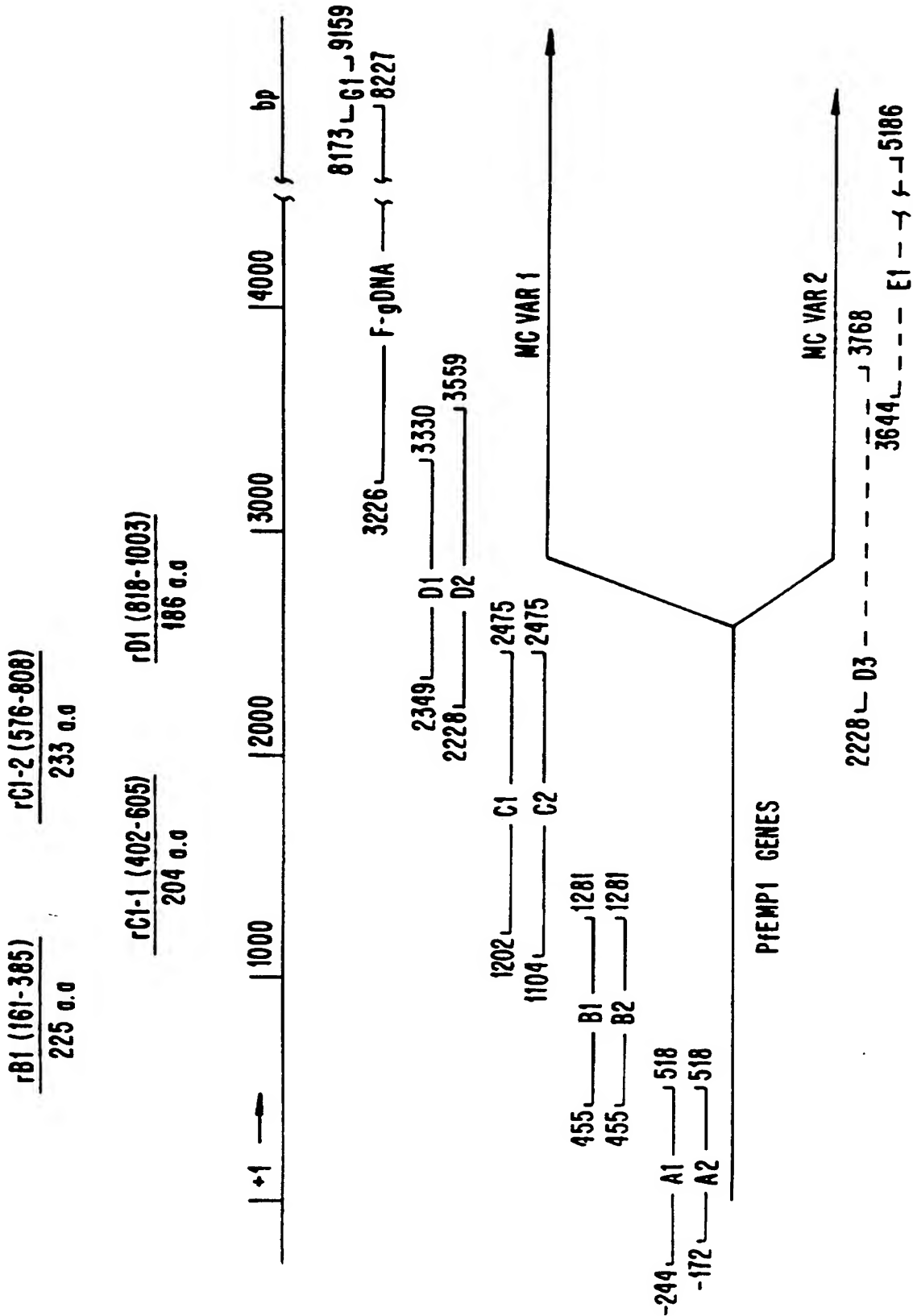



FIG. 1.

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DBL-1-


MGGNGGGGTTKDKAKHALDRIGEEVYKEKVENDAEYKALKGNLQEAKGIGELASSPNPCKLVEDYYNNRLKRKRYPQAN 82  
 RQTVRFSDYGGQCTFNRIKDTKNNDNSIGACAPYRRLLHLC~~LDYNLEKMGKTSTTTKHDLLLDVCM~~AAKYEGDSIKTHYTKHEL 164  
 TNPDTKSQ~~LCTILARSEADIGDIVRGKDL~~YLYGYDDKEKDERKKLENNLIEIFKKIHENLGTODAKDHYKKDEENYYQLREDW 246  
 WTANRSTVWKAITCHAGESDKYFRKTCSSGEWTDKCRCKDEEGKNETNEVPTYFDYVPOYLRF~~FEWA~~ED~~ECRKR~~KKKIEN 328  
 AIKNCRGEKGNERYCDLNGYNCEETARGAEIFVKGDDCHK~~CSVACDRFVKWIDNQRKEEDKQKKKYDEE~~INKTHGTTITGN 410  
 GKINNLYVGHFYKILKKYYPTVDKSLQKLND~~EAI~~CKKPPNVGNEKASTVDFNNEVNTTFSHTTYCEACPWCQAQKEKNGGW 492  
 KAKEKSCAKKKERIFNKENSTDIKILTP~~EKGRSKTLEKLTFC~~KDGQIKNDIWKCHYDDNGTDDQTDSDNCVLGDWGNLT 574  
 KEDKIMSYNAFFWMVVDMLIDSIKWRDEHGR~~CINKDKGKTCIKGCNKKCICFQK~~WVEQKKTEWGKIKDHF~~RKQKDIP~~KDWT 656  
 HDDFLQTLMLKDLLLEIIQDTYGDANEIKRIEALLEQAGVG~~GIDFAALAGLYTKGFVAEKD~~TTIDKLLQHEQK~~EADKCL~~KTH 738  
 TDDTCPPQEDRSVARSESATVSPPADPKATEEVDANASSDDEDDFEEEEEEEEEEVQEEKTDESATEAVAPSPP 820  
 GTTQDGVK~~PASQEDDV~~KVCSIVDKALKGKLDDA~~CTLKYGKTAPT~~SNKQIPS 902  
 DBL-2-  
 -----DTKSVATTGSDTTGSGSICVPPR 894  
 BRKLYVGKLHDWAGGETTEAKSQETSGGQKTPSGNESSPSEKLPQGPTPETTKETPESLLHAFVSP~~RLRRFLPWHK~~FKEQ 984  
 BRKLYVGKLHDWAGGETTEAKSQETSGGQKTPSGNESSPSEKLPQGPTPETTKETPESLLHAFVSP~~RLRRFLPWHK~~FKEQ 976  
 WKAQHAGATGQQTIIIGTLDGGGEETPKLLKTGHIPPDFLRQMFYTLGQYRDI-LVGN~~TDIVVHTSGN-KEDMQIMEA~~IQ 1064  
 WKAQHAGATGLQLPGVTVDSDPD-PQTLKRGNI~~PNDFLRQMFYTLGQYRDI~~CIGGDRDIVGDTIVSITEGESTKKKISK 1057

FIG. 2-1.

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KIEQILPTSGSSPSPRVTTQTQ-----HSVEN-PRKTTWNNENGKKIWEGMVCALTNTDTPSGTAPTQIQEVRTKLWDENS 1138  
 IIEGFLKKQTVTSPSPRDTSSRTPVHPQTSVEKTPQQTWWEANGPHIENGMI~~C~~ALT~~Y~~EDSGAIGQPPQKVEDADKVLEKLP 1139  
 KNPK-IPQYKYDQVKLDDTSDAKTTGSPIPSGEKITPLTDFISRPYPFY~~LEEW~~GETE~~CK~~ERKKRLEKIKEEC-----R 1212  
 NTANGIKWYLKEDNTSSAMPTSSSSSSSGSNDPINTPKLTFEVEIPTFFRYLH~~EW~~GQNE~~CK~~ERMKRLKQIYKE~~CK~~VGENGYGR 1221  
 GDRTGHECHSGDGYDCTRTDAD-RNDKFVDLNCRDCHI~~Q~~CRKYRK~~W~~IDIKFDEYHK~~Q~~EKKYQGEYD-KLT~~K~~DKSSGGDNNCC 1292  
 GRKQKTPQCSCYGEDCEDQLSKYSYD~~T~~VADLECPKCAKH~~C~~RWYK~~K~~WIEKKKDEFTEQEKAFPKQDYVYVNGNKG~~G~~GGDNGFC 1303  
 KDIEKHKSAAVFLKELKHCKNGQTS~~N~~KG~~N~~QEDQLNKLDFDKIPQTFSPSTYCKACPVYGVN~~C~~NGNKRGRGGTNGCTTNNEP 1374  
 ITLKSLSDAAQFLEKLGSK-----KDNSEDNGNDKLNFSQPNETFPATNCKPCSEFKIDCKENGKCKNGGGG--TNETC 1377  
 ENKENDKGAASTISILINDGSTNGATNGTTGTTDET~~L~~K~~E~~CS~~D~~KYAFFGLRKQEWTCQKKYGVNQC-----NL~~T~~NRVNDTYFD 1452  
 NGTTFITSENFKQKGQTAK~~E~~FVMRVSDNNPNNGFDD-LNEACQ~~N~~AGIFK~~S~~IRKDEWECGKVC~~G~~YEVC~~I~~PEKGN~~G~~VTTSGENND 1458  
 CRM-2--  
 KDIVNEFFQRLRYFVHDYNI~~L~~KHKIDPCIKKEKQDKTEHKCINGCNIKCECVRK~~W~~LEIKGNEWGNIKKHYNINSNDDKET 1534  
 QIITIRGLVAHWVQNF~~L~~DDYNIKIKHKISHC-----KNSSEGYTCIKNC-----VEQWISTKRT~~E~~W~~T~~NIKILLNEQYKDNPD- 1529  
 IAYNVKSYFVDQGLFDTDYKKAQKVVEDEKERKKIWGCTGHDECSEKEEKNFITNLISELQDKITSCQNKHNPNGTAC 1616  
 --Y~~N~~VKT-ILQDLQSQIDFNKAIKPCGTLTKFEDSCGING-AESSEKKN~~G~~HEYDAIDCMLNRLQDKIDDCNKNHAQNG---- 1603  
 DBL-3--  
 DFPSP~~T~~PEETDPLDDDTDPDPLDDDDQHT~~E~~QPKFCPPPPPPMTCVEKIAKELRVEAEGKINN~~E~~LKNGKDFNGKCN~~N~~VKKKNG 1698  
 GENQAKCEKHSAP-DEDDDEAIEEENPVTQPNICPKPPEPKAEKGGCEPAEKKEKVEEKEKT~~V~~NTVAKPTEKEAAGDPAG 1684  
 AVIGEESC~~K~~FEQTYENS~~V~~NNINNKKCKDNQNERFKIGQ~~W~~NFKYIGTIRKDL~~C~~IPPRREHM~~C~~LDD~~L~~SMLGRTTISDSSALLKK 1780  
 PAADSEENPEEKAPEPEVETKKDKAPVKPTPASPPPLGLHLYP 1728  
 IQEAAKSERDDIIRKLL~~E~~QNSCDEHRICDAMKYSEADLGD~~I~~IRGRDLWNKNSKQKGLQKRLEYAFINIYNKLQNDKNKYEYD 1862  
 RFKYLOLRSDW~~D~~ANRKH~~I~~WNAMTCNAPDDAKFLKKNPN~~D~~TSGSSSSK~~G~~IMTTHSNCGYDK~~E~~PPDYDIPO~~P~~FR~~W~~MQE~~S~~ES 1944

FIG. 2-2.

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**FIG. 2-3.**

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CRM - 1	607	CI	---	NKDK	-GK	TC	K	GCN	K	GC	FQ	KW	V	EQ	K	T	EWG	K	IK	DHF	RKQ	648		
CRM - 2	1482	CI	K	EKO	DK	TEHK	C	ING	CN	I	K	EC	VR	KW	E	I	KG	EWG	N	IK	H	YNIN	1527	
CRM - 3	2354	NE	KK	AEV	TK	AP	EY	EN	DK	CN	DK	CN	I	SK	Y	I	D	I	FK	N	MY	DSF	DDN	2398

FIG. 3.



FIG. 4B.

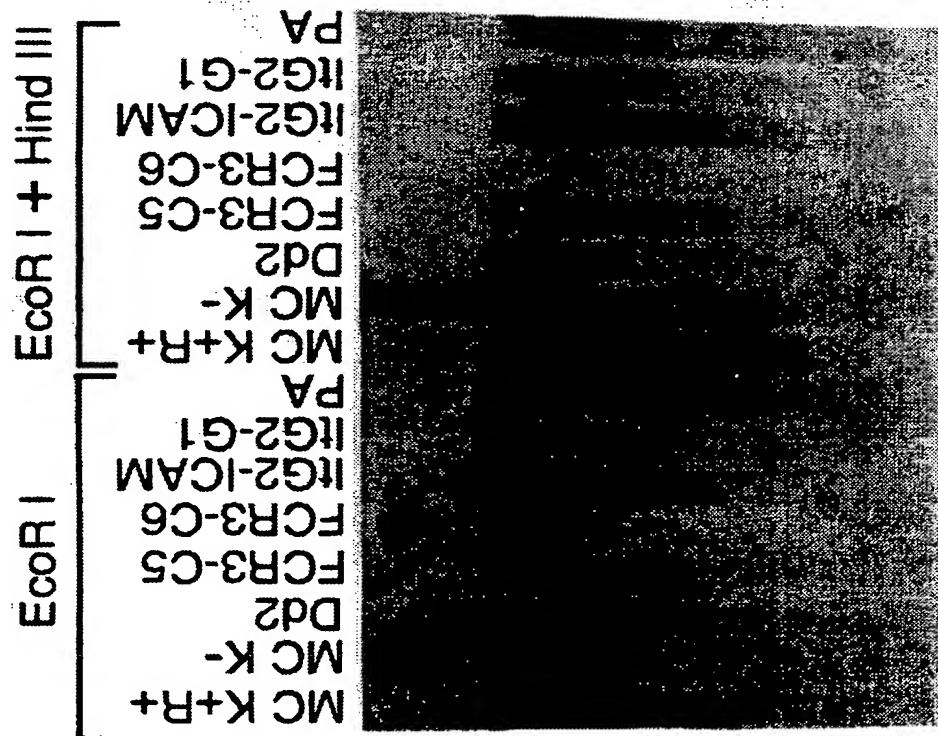


FIG. 4A.

23 -  
12 94 -  
6.5  
1.6 -  
1.0 -

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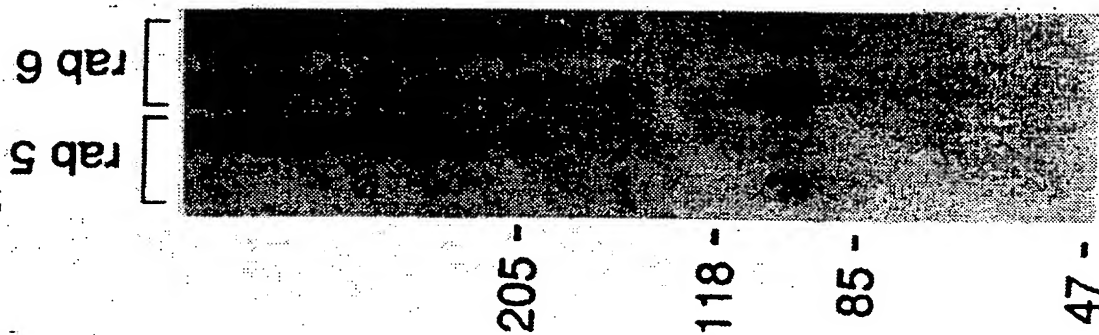


FIG. 5B.

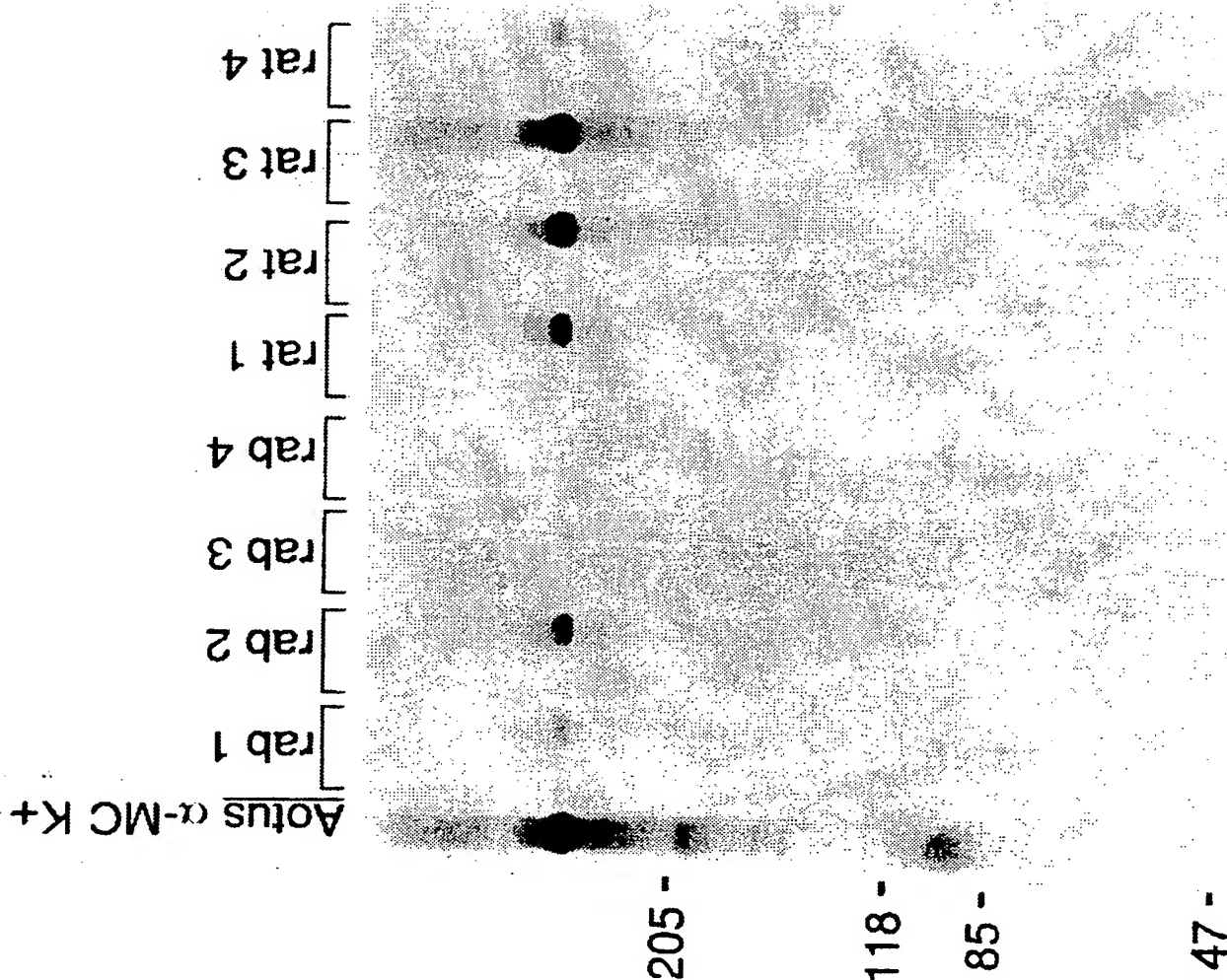


FIG. 5A.



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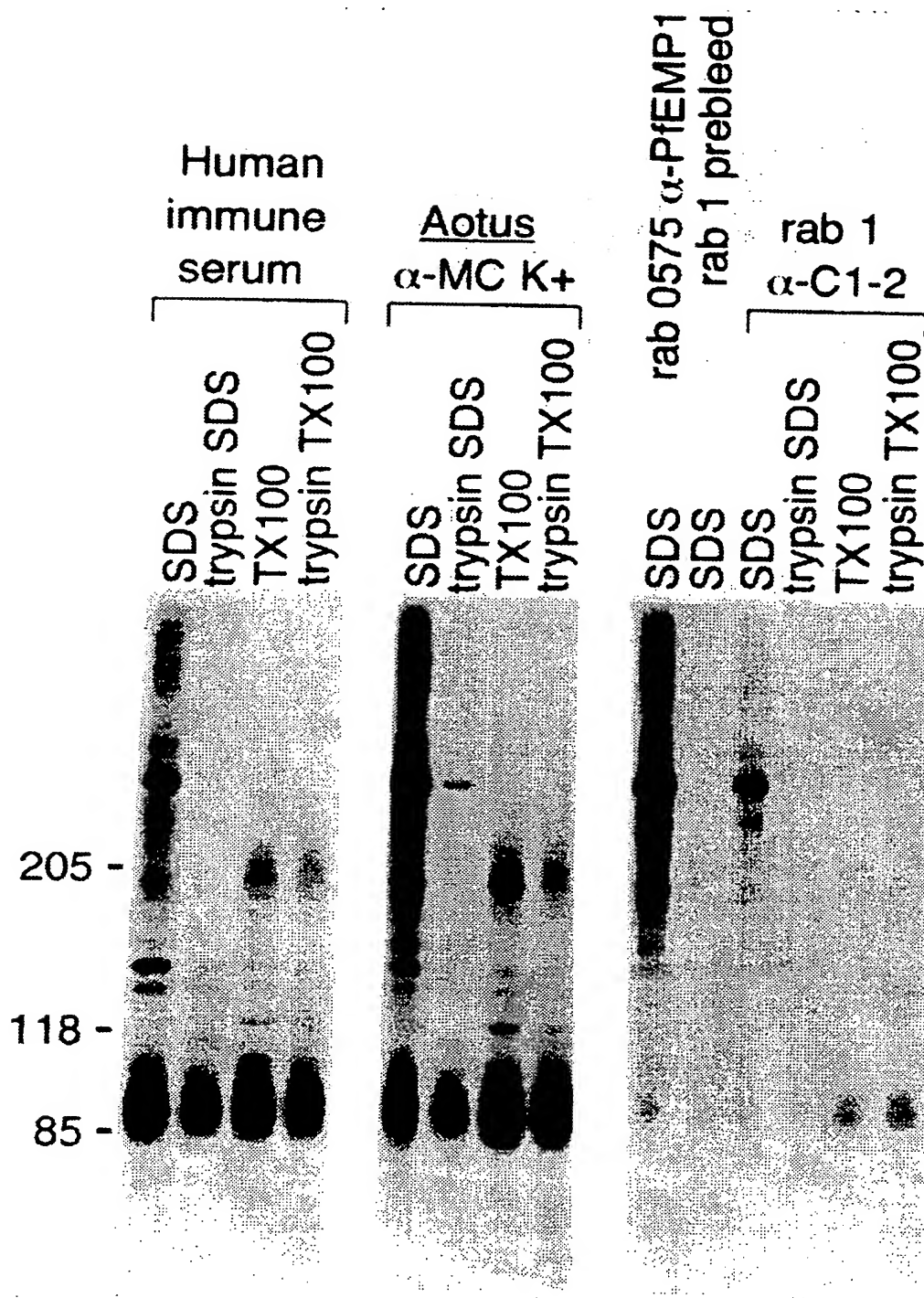
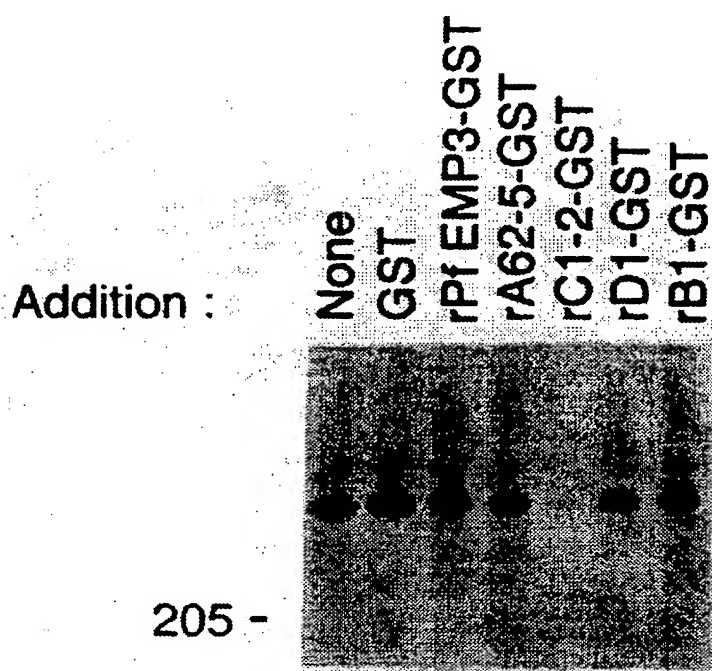
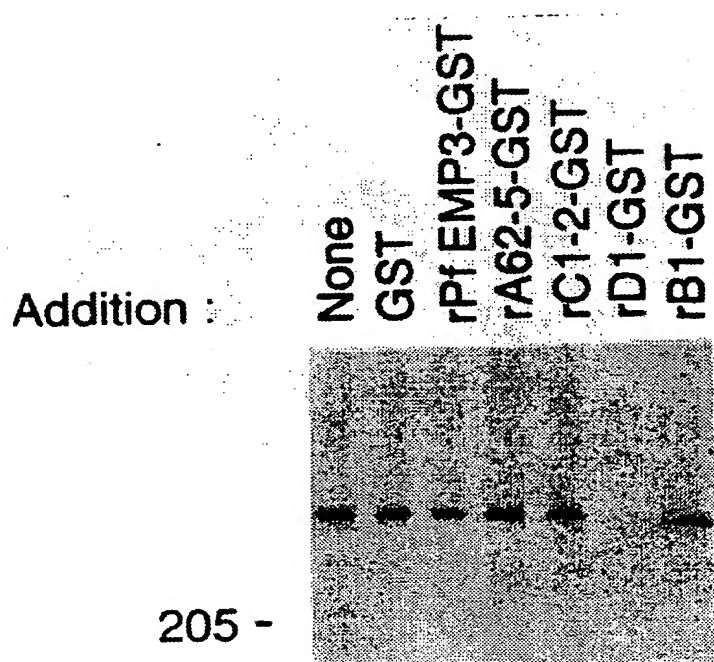
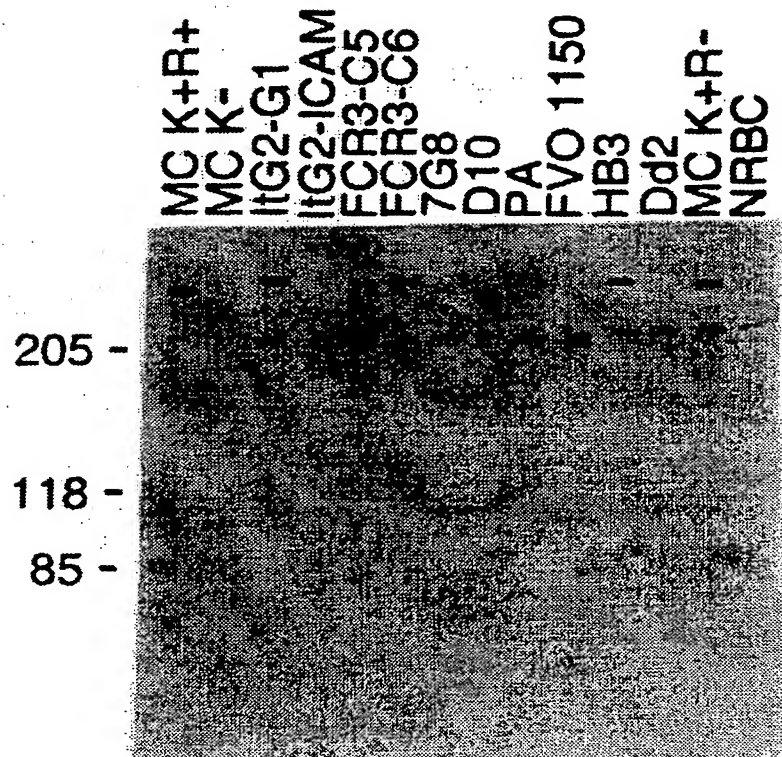


FIG. 5C.

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*FIG. 5D.**FIG. 5E.*

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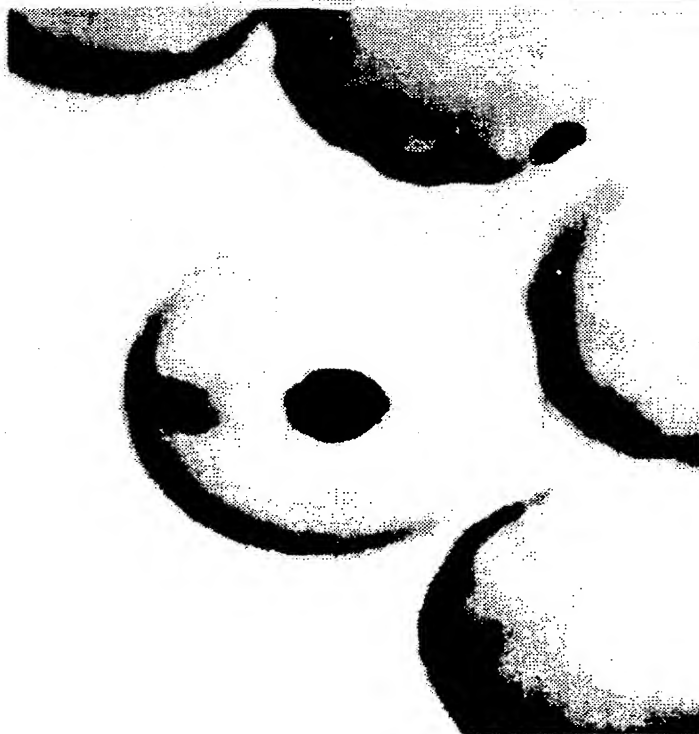


**FIG. 6.**



10  $\mu\text{m}$

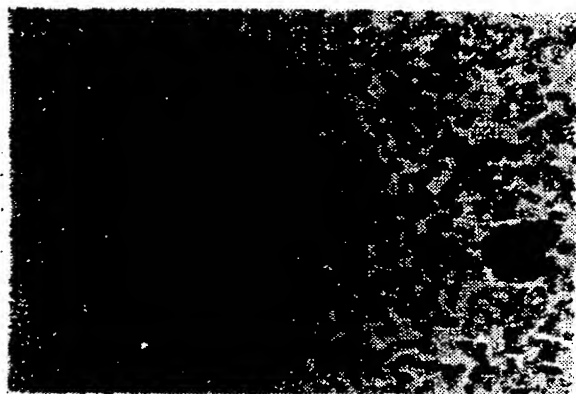
*FIG. 7B.*



*FIG. 7A.*

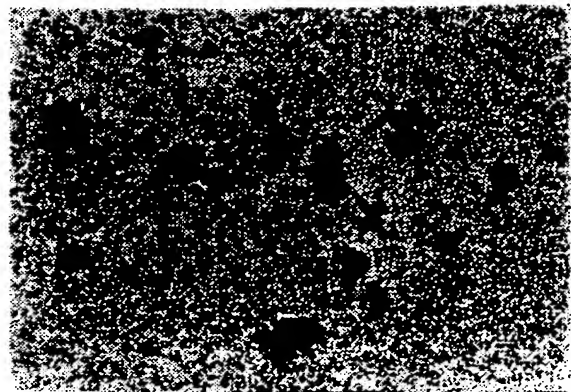
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MC K+ & *Aotus*  
 $\alpha$ -MC K+ (1:5)



*FIG. 8A.*

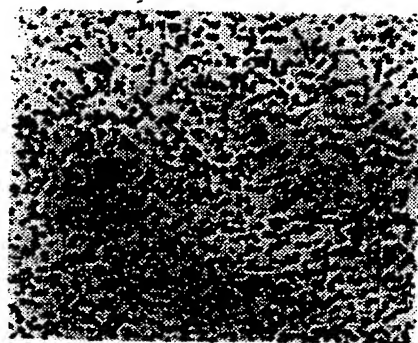
MC K+ & rat  $\alpha$ -MC  
PfEMP1(rC1-2)(1:20)



*FIG. 8B.*

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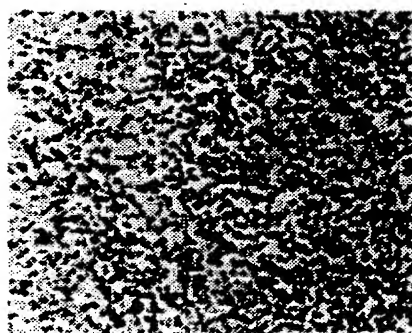


MC K+ & rat  
prebleed (1:5)

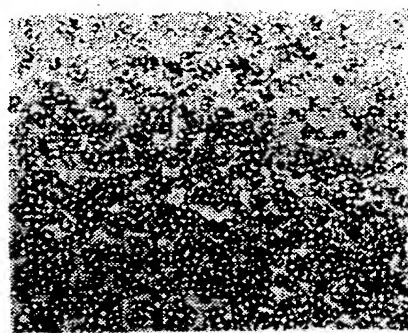


500  $\mu$ m

**FIG. 8C.**



MC K-



ItG2-ICAM1 K+

rat  $\alpha$ -MC PfEMP1 (rC1-2) (1:5)

**FIG. 8D.**

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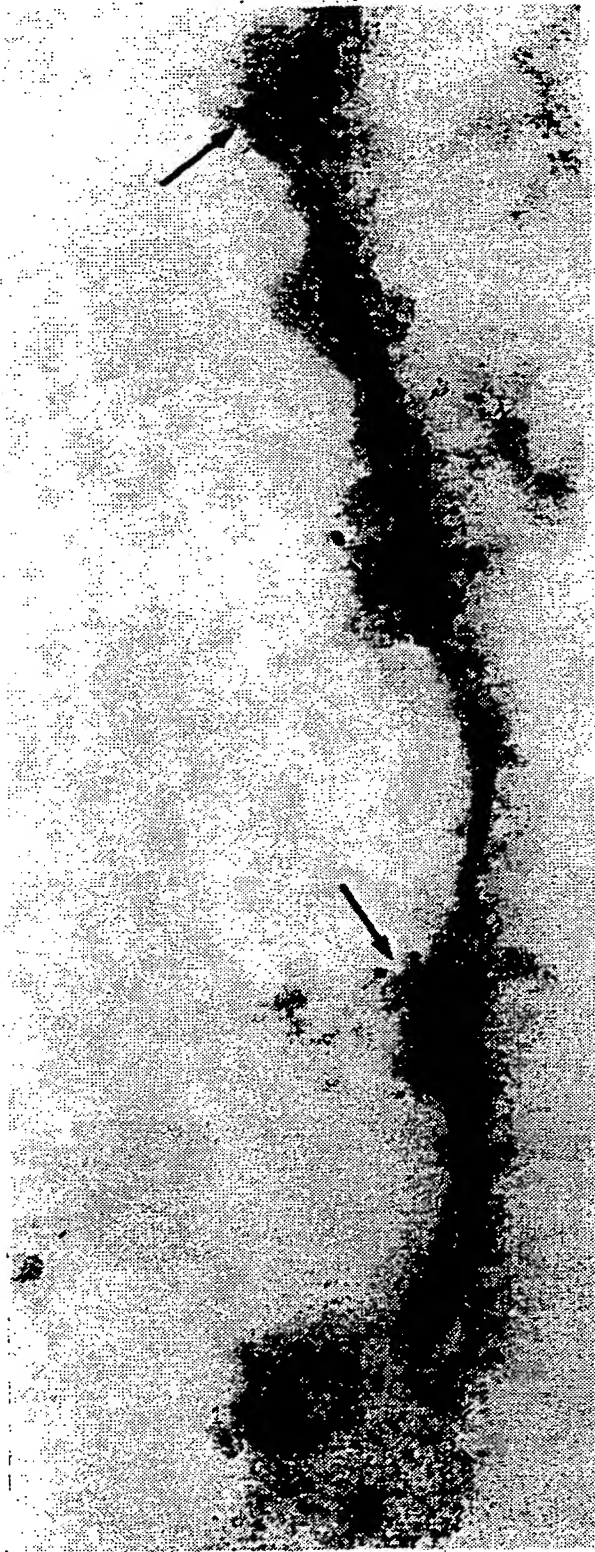


FIG. 9A.

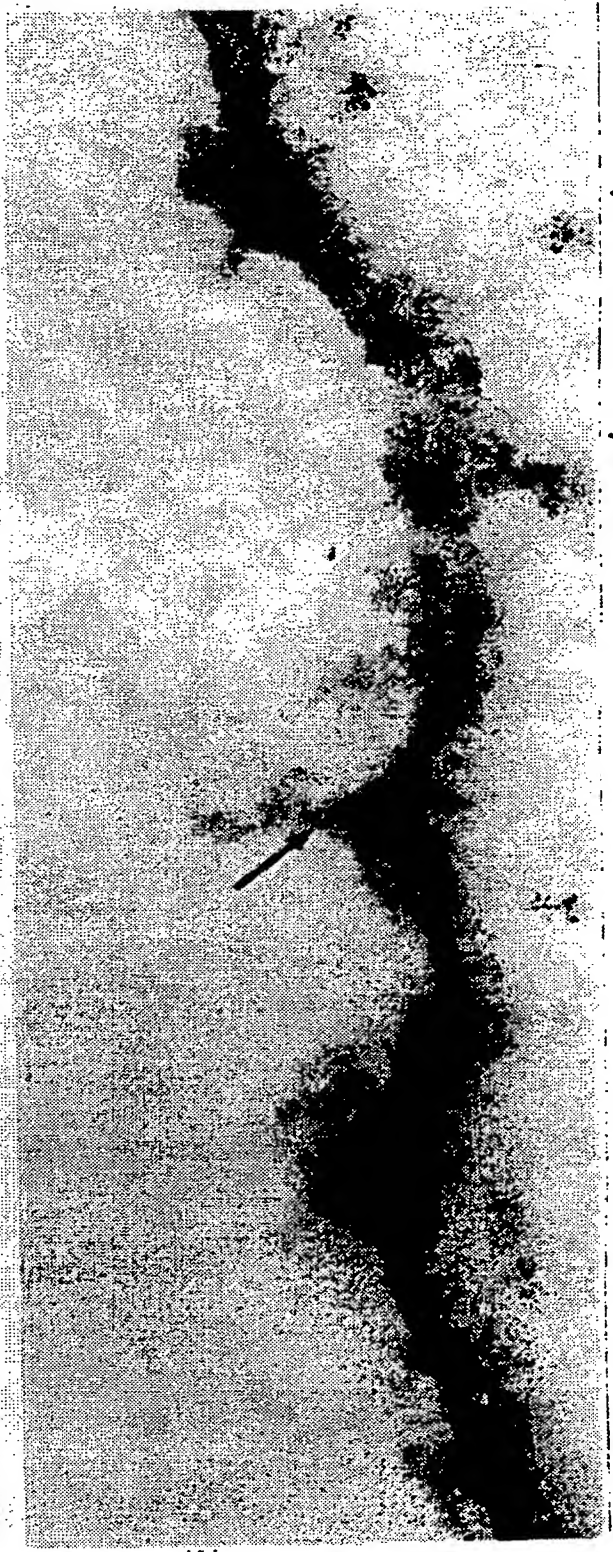


FIG. 9B.

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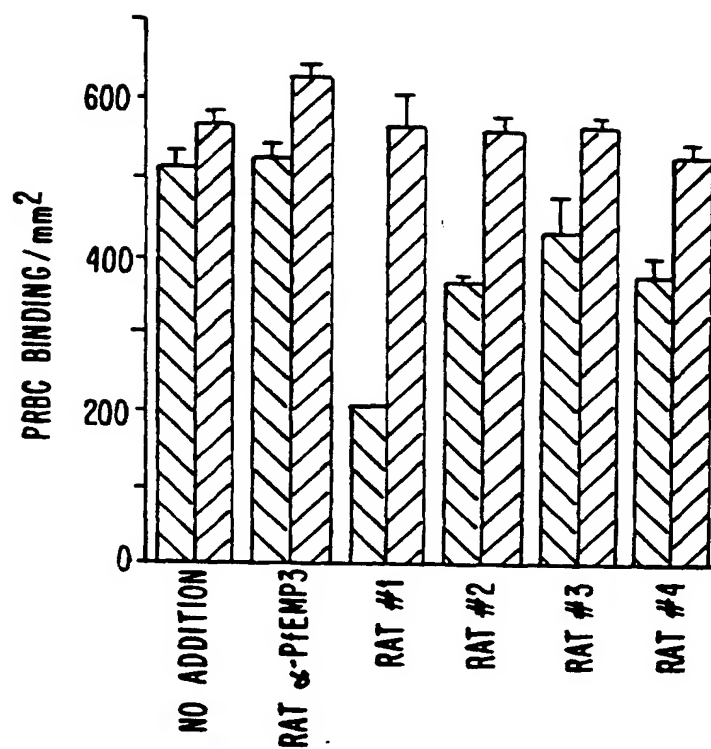


FIG. 10.

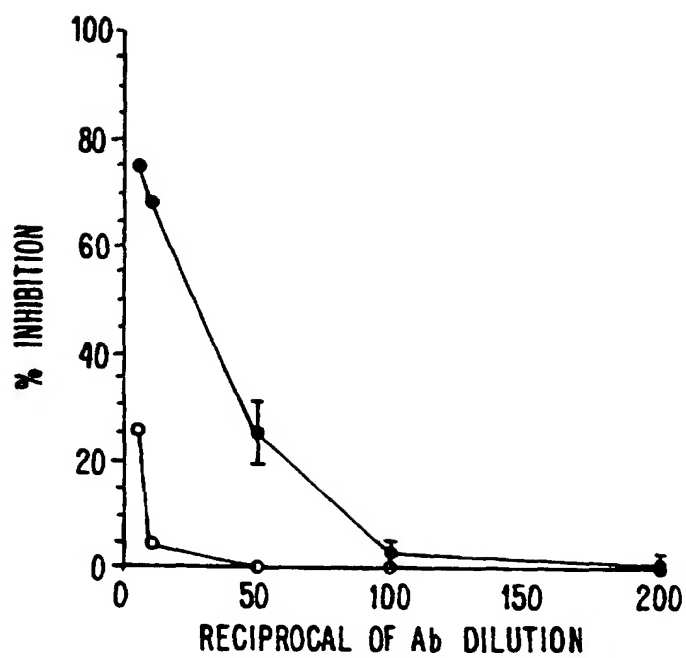


FIG. 11.

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483 A C A A C G A A G C A T G g T T T G T T G T T A G A T G T T T G T A T G G C A G C A A A A T A T G A A G G G A C T C A A T A A A A C A C A T T A T A C A A A A  
 161 T T K H G L L L D V C M A A K Y E G D S I K T H Y T K  
 564 C A T G A A C T A A C T A C T A C T A A A T C T C A A C T A T G T A C T A T A T T A G C A C G C A G T T T G C A G A T A T A G G A T A T T G T A  
 188 H E L T N P D T K S O L C T I L A R S F A D I G D I V  
 645 A G A G G A A A A G A T T T G T A T C T C G G T T A T G A T A A A G A A A A A G A T G A A G A A A A A A T T A G A A A A T A T T G A T A G A A A T T  
 215 R G K D L Y L G Y D D K E K D E R K K L E N N L I E I  
 726 T T C A G A A A A T A C A T G A A A A T T T G G G T A C A C A G A T G C A A A A G A C C A C T A C A A A A A A G A T G A A G A A A T T A T T A T C A A T T A  
 242 F K K I H E N L G T O D A K D H Y K K D E E N Y Y O L  
 807 C G A G A A G A T T G T G G A C A G C G A A T C G C T C C A C A G T A T G G A A A G C C A T C A C A T G T C A T G C A G G A G A A A G T G A T A A A T A T T T T  
 269 R E D W W T A N R S T V W K A I T C H A G E S D K Y F  
 888 A G A A A A C A T G T T C A G G A G A A T G G A C T G A T G A C A A A T G C C G T G T A A G G A C G A G A G G C C A A A A T G A G A C C A A C G A G  
 296 R K T C C S G E W T D D K C R C K D E E G K N E T N E

FIG. 12-2.

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GTCCACACATATTTCGATTATGTGCCACAATATTTAAGATGGTTCGAGGAATGGCCGAAGACTTTTGTCGTAACGAAAA 969

V P T Y F D Y V P O Y L R W F E E W A E D F C R K R K 323  
\* \* \* \* \*

AAAAAAAAATAGAAAATGCTATAAAAAAATTGTCGTGGAGAAAAAGGTAACGAACGATATTGCGGATCTTAATGGATACAAATTGT 1050

K K I E N A I K N C R G E K G N E R Y C D L N G Y N C 350  
\*

GNAAGAANCAGCTAGAGGAGCAGAAAATTTTGTTaAAGGTGATGATTGTCATAAAATGTTCTGTTCCTGTGATCGTTTTGTGA 1131  
<sup>g</sup>

E E T A R G A E I F V K G D D C H K C S V A C D R F V 377  
E \* \*

AAATGGATAGNTAACCAAGAAAAGAATTTGACAAACAAAAAATAATATGATGAAGAAATAAATAAAACTCATGGTACA 1212

K W I D N O R K E F F D K O K K K Y D E E I N K T H G T 404  
\* \* \*

AACAAATAACAACCTGGAAATGGAAAAATTAATAACTTATATGTAGGTCATTTTATATAAAATACTGAAAAAATATTATCCAACA 1293

T I T T G N G K I N N L Y V G H F Y K I L K K Y Y P T 431

GTGTGATAAATCTTACAAAAAATTAATGATGAAGCAATATGTAAAAAACCCACCTAATGTAGGAATGAAAAAGCAAGTACT 1374

V D K S L Q K L N D E A I C K K P P N V G N E K A S T 458

**FIG. 12-3.**

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CTTTGATTTTCAAATAATGAGGTGAACACTACATTTTCTCATACAACATATTGCGAAGCATGTCCGTGGTGGGGCGCAAAA 1455  
 V D F N N E V N T T F S H T T Y C E A C P W C G A Q K 485  
  
 GAAAAAATGGTGGTGGATGGAAAGCTAAAGAAAAAGCTGTGCAAGAAAAaGAAAGAAATATTCAATAAAGAAAAATAGT 1536  
 E K N G G G W K A K E K S C A K K K E R I F N K E N S 512  
 t  
 N  
 ACTTGATATAAAAAATACTTACCCCTGAAAAAGGAAGGTCAAAGACTCTAGAAAAAATTAAAAACCTTCTGTAAAGATGGTCAA 1617  
 T D I K I L T P E K G R S K T L E K L K T F C K D G Q 539  
  
 AAAATTA AAAATGATATTGGAAATGTCATTATGATGATAATGSTACTGATGATCAAACTGATGACAGTAATGACTGTGTA 1698  
 K I K N D I W K C H Y D D N G T D D Q T D D S N D C V 566  
  
 TTAGGAGATTGGGGAAATCTTACAAAGGAAGACAAAAATTATGtCCCTATAATGCTTTTTTTGGATGTGGGTACACGATaTG 1779  
 L G D W G N L T K E D K I M S Y N A F F W M W V H D M 593  
 a  
 T  
 V  
 TTAAATIGATTCTATCAAAATGGAGAGACGAACATGGTAGGTGTATATAAATAAAGATAAGGAAAAACATGTATAAAAGGATGT 1860  
 CRM-1  
 L I D S I K W R D E H G R C I N K D K G K T C I K G C 620  
 a  
 K

FIG. 12-4.

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AACAAAAATGTATGTTTCCAAAAATGGG<sup>9</sup>GTGAACAAAAAAACCGAATGGGGGAAATAAAGACCACCTTTCGCAAG 1941  
N K K C I C F O K W V E O K K T E W G K I K D H F R K 647  
 G  
 CAAAAAGATATTCCAAAGGATTGGACTCATGATGATTTTCTTCAAACTCTTTTGATGAAAGATCTACTTTTGGAAATTATT 2022  
Q K D I P K D W T H D D F L Q T L L M K D L L L E I I 674  
 CAGGATACTTATGG<sup>a</sup>9GATGCAAAATGAAATAAAACGTATTGAGGCACTGTGGACAAGCAGCGCTTGGTGATCGATTTT 2103  
 Q D T Y G D A N E I K R I E A L L E Q A G V G G I D F 701  
 GCTGCTCTTGCTGGTCTGTATACTAAAGGTTTGTGCGCTGAAAAGGACACTACAATTGATAA<sup>t</sup>ATTACTACAACACGAACAA 2184  
 A A L A G L Y T K G F V A E K D T T I D K L L Q H E Q 728  
 N  
 AAGGAAGCCGATAAATGCCCTAAAAACCCACAGATGACACCTGTCCACCACAAGAAGATAGAAGTGTCGCCCGCTCCGAA 2265  
 K E A D K C L K T H T D D T C P P Q E D R S V A R S E 655  
 TCCGCCACCGTCCCCCTACCCACCTGCCGACCCTAAGGCCACTGAGGAAGTCGACGCCCAACGCCCTCCTCAGACG<sup>c</sup>aCGAAGAC 2346  
 S A T V P S P P A D P K A T E E V D A N A S S D D E D 782  
 G

FIG. 12-5.

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**FIG. 12-6.**

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ACTAAACAGGTGCCCGGTACCCCTAGTGGTAAGATACTGGTAGTATTGTGTGCCACCAGGAGGCGAAACTATAC 2721  
T K P G A A G T P S G K D T G S I C V P P R R R K L Y 907  
 \* \* \* \* \*  
T K S V A T T G S D T T G S G S I C V P P R R R K L Y 899  
 \* \* \* \* \*  
 ACAAAAAGTGTGCCACCACTGGTAGTGACACCACCGGTAGTGGTAGTATTGTGTGCCACCACGAGGCGGAAACTATAC 2697  
 GTGGGAAAACTACACGATTGGGCGGTGGTGAGACCACGAGGCGAAGTCACAAGAAACAAGTGGTGCCAAAAGACACCA 2802  
V G K L H D W A G G E T T E A K S O E T S G G O K T P 934  
 \*  
V G K L H D W A G G E T T E A K S O E T S G G O K T P 926  
 \*  
 GTGGGAAAACTACACGATTGGGCGGTGGTGAGACCACGAGGCGAAGTCACAAGAAACAAGTGGTGCCAAAAGACACCA 2778  
 AGTGGTAATGAGTCACCAAGTGAGAAGTTACCACAAGGCCCCACTCCGGAAACGACGAAAGAGACGCCAGAGTCGTCT 2883  
S G N E S S P S E K L P O G P T P E T T K E T P E S S 961  
S G N E S S P S E K L P O G P T P E T T K E T P E S S 953  
 AGTGGTAATGAGTCACCAAGTGAGAAGTTACCACAAGGCCCCACTCCGGAAACGACGAAAGAGACGCCAGAGTCGTCT 2859

FIG. 12-7.

SUBSTITUTE SHEET (RULE 26)

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C T C C T T C C A C G C C T T C G T G A G T C C G C G C G G T T G A G A C G T T T T T T G C C A T G G C A T A A A T T T A A A G A A C A G T G G A A G G C A C A A 2964

I L H A F V S P P R L R R F L P W H K F K E O W K A O 988

I L H A F V S P P R L R R F L P W H K F K E O W K A O 980

C T C C T C C A T G C C T T C G T G A G T C C G C G C G G T T G A G A C G T T T T T T G C C A T G G C A T A A A T T T A A A G A A C A G T G G A A G G C A C A A 2940

C A C G G A G C G G G G G C A C A G G A C A A A C C A T C A T C G G T A C C C T T G A C G G T G G T G A G A A A C C C C G A C A A A C T G T T A 3045

H G A G A T G O O T I I G T L D G G G E E T P D K L L 1015

H G A G A T G L O L P G V T V D D S D P D - P O T O L 1006

C A C G G A G C G G G G C A C A G G A C T A C A A C C C G G T G T C A C T G T T G A T G A T A G T G A C C C T G A T - - - C C C C A A A C A C A A T T A 3018

A A A A C T G G T C A T A T A C C C C C G A T T T T T G C G T C A A A T G T T T A T A C T T T A G G G G A T T A T A G A G A T A T T - - - T T A G T G G G T 3123

K T G H I P P D F L R O M F Y T L G D Y R D I - L V G 1041

K R G N I P N D F L R O M F Y T L G D Y R D I C I G G 1033

A A A C G T G T A A C A T C C C C A A C G A T T T C T T G A G G C A A A T G T T T A T A C G T T A G G A G A T T A T A G A G A T A T A T G C A T A G G T G G T 3099

FIG. 12-8.

SUBSTITUTE SHEET (RULE 26)



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AACACGGATATAGTGGTTCATACAAAGTGGTAAC---AAGGAGGACATGGAAATAATGGAGGCAAT'ACAAAAGAAAATAGAA 3201  
N T D I V V H T S G N - K E D M O I M E A I O K K I E 1067  
D R D I V G D T I V S I T E G E S T K K I S K I I E 1060  
 GACCCGGATATCGTTGGAGACACTATTGTTAGCATCACAGAGGTTGAAGCACAAAAAAAATAATCGAAAAATAATAGAA 3180  
 CAAATTC'TCCAACTAGTGGTAGCTCCCCCATCTCCTCCTCGTGTCCACCCAAACCCAA-----CATAGT 3264  
Q I L P T S G S S P S P R V T O - - - - H S 1088  
G F L K K O T V T S P S P R D T S S R T P V H P O T S 1088  
 GGAT'TTTTAAAGAAACAACACTGTCACATCCCCCTCTCCTCGTGTGACACATCTAGTCGCACACCTGTCCACCCCACTAGT 3261  
 GTCGAAAAC---CCACGTAAACCTGGTGGAAATGAAAATGGCAAGAAATATGGGAAGGTATGGTATGTGCACTAACATAC 3341  
V E N - P R K T W W N E N G K K I W E G M V C A L T Y 1114  
V E K T P O O T W W E A N G P H I W N G M I C A L T Y 1114  
 GTCGAAAAAACCCCCCAACAACCTGGTGGGAAGCAAAATGGTCCTCATATCTGGAATGGAATGATATGTGCTTTAACCTAT 3342

**FIG. 12-9.****SUBSTITUTE SHEET (RULE 26)**

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AATACAGACACCGAGTGGCACAGCACCAACACAAATTCAGGAAGTGCGCACAAAAC<sup>C</sup>TTtGGGACGAAACACAGCAAAAAC 3423  
H T D T P S G T A P T O I O E V R T K L W D E N S K N 1141  
E D S G A I G O P P O K V E D A D K V L E K L K P N T 1141  
 GAAGA'TAGTGGCGCAATAGGACAACCAACCAACAAAAGTTGAAGATGGGGACAAAGTTTGGAAAAA<sup>R</sup>ACTCAAAACCAATACG 3423  
 CCAAA--ATCCCCCAATACAAATACGACCAAGTCAAACTAGATGATACAAAGTGATGCCAAAACCAACCGGCTCCCCCaTC 3501  
P K - I P O Y K Y D O V K L D D T S D A K T T G S P I 1167  
A N G I K W Y L K E D N T S S A M P T S S S S S G S 1168  
 GCGAATGGAATAAAGTGTACTTGAAAGAAGATAATACCTCCTCCGCCATGCCTACCTCCTCCTCATCCTCTAGTGGTAGT 3504  
 CCTAGTGGTGA AAAATCACCCTCCCTCACCCTGACTTTATATCAGCCCCCTTACTTCCGATACCTTGAAGAATGGGGAGAA 3582  
P S G E K I T P L T D F I S R P P Y F R Y L E E W G E 1194  
H D P I N T P K L T E F V E I P T F F R Y L H E W G C 1195  
 AAGACCCCATCAACACCCCAAAATTGACAGAAATTGTGGAATAACCTACGTTTTTCGTTACCTGCACGAATGGGGTCAA 3585

FIG. 12-10.

SUBSTITUTE SHEET (RULE 26)

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ACAATTGTTAAAGAGAGGAGGAAAGATTGGAGAGATAAAGGAGGAGTGT-----CGTGGC 3639  
 T F C K E R K K R L E K I K E E C - - - - R G 1213  
 \* \*  
 N F C K E R M K R L K O I Y K E C K V G E N G Y G R G 1222  
 \* \*  
 AATTGTTAAAGAGAGAGAAATGAAGCGGTTGAAACAAATTTATAAAGAGTGTAAGGTAGTGAAATGGTTATGGTCGTGGT 3666  
 GATTAGGACAGGTCACGAGCATTGTAGTGGGATGGTTATGACTGTACACGAACAGATGCTGAT---CGTAACGATAAAATTT 3717  
 D R T G H E H C S G D G Y D C T R T D A D - R N D K F 1239  
 R K O K T P O C S C Y G E D C E D O L S K Y S Y D - V 1249  
 CGTAACCAAAAAACCCCAATGTAGTTGTTATGGGGAAGATTOTGAAGATCAGCTTAGTAAGTATTTCATATGATACTGTT 3747  
 GTGGATTTTAAATTGTCGTGATTGTCAATATACAATGTAGAAAAATATAGAAAAATGGATAGATATAAAATTCGATGAATATCAT 3798  
 V D L N C R D C H I O C R K Y R K W I D I K F D E Y H 1266  
 \* \* \* \* \*  
 A D L E C P K C A K H C R W Y K K W I E K K D E F T 1276  
 \* \* \* \* \*  
 GCTGATTTAGAATGTCCAAAAATGTGCCAAACATTGTAGATGGTATATAAAAAATGGATAGAAAAAAGACGAATTACT 3828

FIG. 12-II.

SUBSTITUTE SHEET (RULE 26)

27/53

AAGGAAAGAAAGAAATATCAAGGGGAATATGAT---AAATTAAACAAAAGATAAAATCTAGTGGTGGTGATAATAATTGTTGT 3876  
K O E K K Y Q G E Y D - K L T K D K S S G G D N N C C 1292  
E O E K A F P K Q K D Y Y V N G N N K G G D N G F C 1303  
 GAACAAGAAAGGCATTTCCTAAACAAAAGACTATTACGTAAATGGAAATAATAAGGGTGGTGGTGATAATGGATTGTTGT 3909  
 AAGATATAGAAAAACATAAGTCTGCTGCAGTTTTTTGAAAGAATTGAAACATTGCAAAAATGGTCAAACCTAGTGAGAAT 3957  
 K D I E K H K S A A V F L K E L K H C K N G Q T S E N 1319  
 I T L K S L S D A A Q F L E K L G S C K - - - - K 1324  
 A'AAACACTAAAAAGTCTCTGACGCTGCACAATTTTGTAGAAAAGTTAGGATCATGTAAA-----AAG 3972  
 AAGGTAATCAGGAGGATCAACTTAATAAACTAGATTTTGATAAAATTCCTCAGACATTTTCTCCTTCAACGATTTGTAAA 4038  
 K G N Q E D Q L N K L D F D K I P Q T F S P S T Y C K 1346  
 D H S F D N G N D K L N F S Q P N E T F V P A T N C K 1351  
 GATAATAGCGAGGATAATGGAAATGATAAATTAATTTTAGTCAACCAAATGAACACATTTGTACCTGCAACAAATTTGTAAA 4053

FIG. 12-12.

SUBSTITUTE SHEET (RULE 26)

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GGGTTGTCCTGTATTATGGAGTTAATTGTAATGGTAATAAACGTGGTAGAGGTGGTACAAATGGATGTACAAACGAATAATGAA 1119  
A C P V Y G V N C N G N K R G R G G T N G C T T N N E 1373  
P C S E F K I D C C K E N G K C K N G G G G - - T N E T 1376  
CCATGTTCTGAATTTAAATAGATTGTAAAGAAAATGGTAAATGCAAGAATGGTGGTGGT-----ACAAACGAGACC 4128  
CCTGAAAAATAAGGAAAATGACAAAGGAGCTGCAAGTACTATCTCTATACTGATAAATGATGGTTCTACTAATGGTGCTACT 4200  
P E N K E N D K G A A S T I S I L I N D G S T N G A T 1400  
C N G T T F I T S E N F K Q K G Q T A K E F V M R V S 1403  
TGCAATGGAACAACCTTTATTACTTCAGAAAAATTTTAAACAAAGGACAGACTGCTAAAGAATTGTGTTATGCGTGCAGT 4209  
AATGGTACGACTGGTACTACTGATGAACACATTAAAGAGTGTCTCTGATAAATATGCGTTCTTTAAAGGTTTAAAGAAAACAA 4281  
H G T T G T T D E T L K E C S D K Y A F F K G L R K Q 1427  
D I I H P N G F D D - L N E A C Q N A G I F K S I R K D 1429  
GATTAAACAATCCAAAATGGATTGTGATGAT----TTAAATGAGGCTTGTCAAAATGCAGGTATATTTTAAAGTATTAGAAAAGAT 4287

*FIG. 12-13.*

SUBSTITUTE SHEET (RULE 26)

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GAA'TGGACATGCCAAAGTATGGAGTAAATCAATGT-----AATCTTACAAATCGTGTGAATGATACATAT 4350  
 E W T C Q K K Y G V N Q C - - - N L T N R V N D T Y 1450  
 E W E C G K V C G Y E V C I P E K G N G V T T S G E N 1456  
 GAA'TGGGAATGTGGCAAGTATGTGGTTATGAGGTATGTATACCGGAAAGGGTAATGGGGTAACAACCAAGTGGGGAAC 4368  
 TTTGATAAAGATATTGTATTTAATGAATTTTTCAGCGATGGTTAAGATATTTTGTACACGATTATAATATATTGAAACAC 4431  
 F D K D I V F N E F F Q R W L R Y F V H D Y N I L K H 1477  
 N D Q I I T I R G L V A H W V Q N F L D D Y N K I K H 1483  
 AATGATCAAAATTATAACAATTAGAGGTTTGGTTGCACATTGGGTACAAAATTTTGTAGACGATTATAATAAAATTAAACAT 4449  
 AAAA'TTGGATCCATGATAAAAAAGGAAAGCAGGATAAAACAGAACACATAAATGTATTAA'TGGATGTAACATTAAATGTGAA 4512  
 K I D P C I K K E K O D K T E H K C I N G C N I K C E 1504  
 K I S H C - - - K N S S E G Y T C I K N C - - - 1501  
 AAAA'TTTCACATTGT-----AAGAATAGTAGTGAAGGATACACATGTATAAAAAATTGT----- 4503

CRM-2 →

FIG. 12-14.

SUBSTITUTE SHEET (RULE 26)

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TCCTCTGAGAAAATGGTTAGAAAATAAAGGGAAACGAATGGGGAATATAAAAAACATTATAACATAAAATTCAAAATGATGAT 4593

C V R K W L E I K G N E W G N I K K H Y N I N S N D D 1531

- V E Q W I S T K R T E W T N I K I L L N E Q Y K D N 1527

---GTAGAACAAATGGATAAGTACGAAAAGGACAGAAATGGACAAATATAAAAAATCCTTCTTAATGAGCAATATAAAGATAAT 4581

AAGGAGACTATTGCTTATAACGTTAAAAGTTATTTTGTGATCAGGGACTCTTTGACACCGATTATAAGAAAGCCCAAAAG 4674

K E T I A Y N V K S Y F V D Q G L F D T D Y K K A Q K 1558

P D - - - Y N V K T - I L Q L D Q S Q I D F N K A I K 1550

CCTGAT-----TACAATGTCAAAACC---ATTTGCAGGACTTGCAATCTCAAATTGATTTTAACAAAGCTATAAAA 4650

GTGGTTGAAGACGAAAAGGAAAAGAAAATAATGGGGATGTACTGGTCACGATGAGTGCAGCGAGAAAGAGAAAGAGAG 4755

V V E D E K E R K K I W G C T G H D E C S E K E K E E 1585

P C G T I, T K F E D S S G I N G - A E S S E K K N G H 1576

CCCTGTGGTACTTTAACGAAGTTCGAGGATTTCGTGTGTTAATGTC---GCTGAGAGCTCAGAAAAAAAATGGTCAT 4728

FIG. 12-15.

SUBSTITUTE SHEET (RULE 26)

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AAATGAAAAATTTTATAACAAATTTAATTTCTGAACCTTCAAGACAAAATAACATCTTGTCAAAAACACACCAACCCTAATGGC 4836

N K N F I T N L I S E L Q D K I T S C Q N K H N P N G 1613

E Y D A I D C M L N R L Q D K I D D C N K N H A Q N G 1603

GAGTATGATGCTATAGATTGTATGCTTAATAGACTTCAAGATAAAAATTGACGACTGCAATAAGAAACCACGCCCAAAATGGT 4809

AAAAACAGCTTGTGATCCATTCCCCCTCCCCACACCCCGAAGAAACCGACCCCCCTTGACGACGACACACCCGACCCCTTGAC 4917

K T A C D P F P S P T P E E T D P L D D D T P D P L D 1639

- - - G E N Q A K C E K H S A P - D E D D E A I E 1625

-----GGCGAAAACCAAGCAAGTGTGAAAAACACTCCGCCCT---GATGAAGATGACGATGAAGCCATTGAA 4875

GACGACCAGCACACAGAACAGCCAAAGTTTGTCCACCACCTCCACCGCCAATGACGTGTGTGAGAAAATAGCGAAAGAA 4998

D D Q H T E Q P K F C P P P P P P M T C V E K I A K E 1666

E E N P V T Q P N I C P K P P E P K A E E K G C E P 1652

GAGGAAAACCCAGTAACACACACCCCAACATTGTGCCAAAACCCACCAGAACCAAAAGCAGAGGAAAAGCGGTTGTGAACCA 4956

FIG. 12-16.

SUBSTITUTE SHEET (RULE 26)



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TTCTAGAGGCAGGGAAGATAAATAATGAATTGAAGGGAATGAAAAGATTTTAATGGAATGTAATAATGTG 5079  
 L K V E A E G K I N N E L K G N G K D F N G K C N N V 1693  
 A F K K E K V E E K E K T V N T V A K P T E K E A A 1679  
 GCAGAGAAAAAAGTGAGGAAAAAGAGAGAAAAACAGTGAATACAGTGCGGAAACCTACAGAGAAAGAGCAGCT 5037  
 AAGAAAAAATGGTGCTGTTATTGGGGAAGAGTCATGCAAAATTCGAACAAACATATGAGAATTCAGTAAATAACATAAAT 5160  
 K K K N G A V I G E E S C K F E O T Y E N S V N N I N 1720  
 G D P A G P A A D S E E N P E E E K A P E P E V E T K 1706  
 GTTGACCCAGCGGACCAGCAGCAGATAGTGAGGAAAAACCCCGAAGAAAGAACCCGGAACCCAGAAAGTGGAACAAAA 5118  
 AAGAAAATGTAAAGATAATCAAAACGAACGTTTCAAAAATAGGACAAAAATGGAATTTCAAGTATATAGGTACAAATACGAAAG 5241  
 H K C K D N O N E R F K I G O K W N F K Y I G T I R K 1747  
 K D K A P V K P T P A S P P - G L H L Y P 1728  
 AAGACAAAGCCCCAGTCAAACCAACACCAGCATCTCCACCCCCCGCTGGGCCCTCCATCTTACCCCCC 5186  
 GATTTATGTATTCCTCCAAGACGAGAAACATATGTGCTTAGATGATTAAAGTATGTTAGGGCGTACTACTATTAGTGATAGT 5322  
 D L C I R P R R E H M C L D D L S M L G R T T I S D S 1774

SUBSTITUTE SHEET (RULE 26)

FIG. 12-17

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AGTGGCTTTTACTAAAAAAGATTCAAGAAGCAGCAAGAGAGTGACATTATAAGAAAAATTATTAGAACAAAAATTCA 5403  
S A L L K K I O E A A K S E R D D I I R K L L E O N S 1801  
 TGTGATGAGCATCGAATTTGTGATGCTATGAAATATAGTTTGTGCTGATTAGGAGATATTATAAGAGGAAGAGATTATG 5484  
C D E H R I C D A M K Y S F A D L G D I I R G R D L W 1828  
 \* \* \* \* \*  
 AATAAAANTAGCAAAACAAAAGGATTGCAAAAAAGATTAGAGTATGCGTTTATAAATATATATAATAAATTGCCAAAAACGAT 5565  
N K N S K O K G L O K R L E Y A F I N I Y N K L O N D 1855  
 AAGAATAATATAGAGAAAGATAGACCAAAATATTTACAATTACGTTCTGATGGTGGGATGCTAATAGAAAAACATATATGG 5646  
K N K Y E K D R P K Y L O L R S D W W D A N R K H I W 1882  
 \* \* \* \* \*  
 AATGCTATGACTTGTAATGCTCCTGATGACGCTAAATTTTAAAAAAAATCCAAATGATACCTCAGGAAGTTCATCTTCA 5727  
N A M T C N A P D D A K F L K K N P N D T S G S S S 1909  
 \*  
 AAGGGCATTATGACTACTCATTTCTAATTGTGGATATGATAAAGAACCCACCTGATTATGATTATATTCCTCAACCTTTTCGT 5808  
K G I M T T H S N C G Y D K E P P D Y D Y I P O P F R 1936  
 \* \* \* \* \*  
 TGGATTGCAAGAATGGAGTGAAAGTTTTCGCAAAATTATTAAATGAAGAAATGGAACAATTTGAAAAAACATGTGGAGAATGT 5889  
W M O E W S E S F C K L L N E E M E O F E K T C G E C 1963  
 \* \* \* \* \*

FIG. 12-18.

SUBSTITUTE SHEET (RULE 26)

AAAAAAAAAATAGTATTACATGTGAAGATGACAGAAATGGAACGAACTGTGAAAACTGCAAAAAATCAATGTGAAAAATATAAAA 5970  
 K K N S I T C E D D R N G T N C E N C K N O C E K Y K 1990  
 AACITATTCAATAATTGGAAAATTAGGATTCGATAAAATATAAAGAAAATATATAATGAAAATATATAATAAGGACAGCAAA 6051  
 K L I H N W K L G F D K Y K E I Y N E I Y N N K D S K 2015  
 ATTAATTCAAAATGAATATTTTAAAAAATTTTAGAAAAAACTAAAGATAAAATGCAAGAACTAAACAGTTCTGATAAAATGT 6132  
 I N S N E Y F K K F L E K L K D K C K E L N S S D K C 2044  
 ATTGATGAAGCAACCCACTGCACAAAAATACAAAATTTAGTAATAGCGAAAAATAAAAAATCATAATAATTATGCTTCAAAAAAT 6213  
 I D E A T H C T K Y K F S N S E N K N H N N Y A F K N 2071  
 CCCCCAAAAGAATATGAAAAAGCAATGTGATGCACCGGATCCATTAGATAAATTGTCCTAAAGATAGTGCCACATAT 6294  
 P P K E Y E K A C K C D A P D P L D N C P K D S A T Y 2098  
 GAAAAGGCGGTGAACACTTTGCTTCCTACAAAAATTATGTGAAAGTAAAAACAATTTAATAATGATGATAGTTGGGATACT 6375  
 DBL-4 →  
 E K A C N T L L P T K L C E S K T F N N D D S W D T 2125  
 TCTTTTGTGCAAAACATCACCTCGTGACAATACAGGTGTTTAGTTCACCTAGAAAGGAGACAAATATGTCTTAAAAAATATA 6456  
 S F V O T S P R D N T G V L V P P R R R O I C L K N I 2152

**FIG. 12-19.**

**SUBSTITUTE SHEET (RULE 26)**

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AC"TAACAAGTTACGTTCTATAGAAAAAATAGATGATTTTCAAGCCGAACTTATGACATCTGCCTACAATGAAGGGAATTA 6537  
T T K L R S I E K I D D F K A E L M T S A Y N E G K L 2179  
 TTATGTGAACATATAAAAAGGATAGAGATGTTACTTTGCAAGCAATGAAATATAGTTTTTATGATTATGGAGATATAGTT 6618  
L C E L Y K K D R D V T L O A M K Y S F Y D Y G D I V 2206  
 AAAGGAACCTGATCTGATAAGTACTGCACCATTGGATAAATTAAAAACAATAAATGTACTTCTTAAAGGAGATGGAACT 6699  
K G T D L I S T A P L D K L K T K L N V L L K G D G T 2233  
 AATGAAATATAAAGAAGATCGCGGAAAGTGGTGGACTGAAAAATAGAACACGAGTTTGGCATGCTATGTTATGCGGATATAAA 6780  
N E I K E D R G K W W T E N R T R V W H A M L C G Y K 2260  
 GCTGCAGGAGGAAAAAATTGAAGAAAGAGATTGTTTCATTACCTGATGATAATACTCATCAGTTTCTTCGATGGTTTCGTGAA 6861  
A A G G K I E E R D C S L P D D N T H O F L R W F R E 2287  
 TGGAGCGAACAATTTTGGCGTAAACGACAAAAATTATTAACGAGGTAAGGAAAGGAAATGTCATCAGCGCAATGTATTATT 6942  
W S E H F C A K R O K L F N E V K R E C A S A O C I I 2314  
 \* \* \* \* \*  
 CAGTAATGGAACATATTGATCCGCCCTGTTGTGAAGAGGCGGTGACTCAATATAGGGATTATATTAACAAGGAAGATACAAGAG 7023  
E Y G T I D P P V C E E A C T O Y R D Y I T R K I O E 2341  
 \* \* \* \* \*

**FIG. 12-20.****SUBSTITUTE SHEET (RULE 26)**

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FIG. 12-21.

**SUBSTITUTE SHEET (RULE 26)**

[illegible]

**FIG. 12-22.**

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A T A C C C A T C C T A C C A C G T C A T A A T G T G G A A G A A A A C C T T T T A T T A T G T C T A T T C A T G A T G G A T T T A T A T A G T G 8724

N T H P T T S R H N V E E K P F I M S I H D R D L Y S 2666

G A G A A G A A T A T A G T T A A T A T G T T A A T A T G T T A A T T C C A A T A A G T G C T A G A A A T G G T A A C T A T A T A G T G G T A T A G 8805

G E E Y S Y N V N M V N N D I P I S A R N G N Y S G I 2693

A T T T A A T T A A T G A T T C G T T A A A T A G T A A A G G T G G A T A T A T A T G A T G A A T T G T T G A A A C G A A A A A C G A A T T A T T T G 8886

D I I N D S L N S N K V D I Y D E L L K R K E N E L F 2720

G G A C A A A T C A T A C G A A A A A A A T A C A T C A C C A A T A G T G T G C A A A A A A T A C A A A T A C T G A C C C C A T C C A C A C C A A C T A A 8967

G T N H T K K N T S T N S V A K N T N T D P I H N Q L 2747

A T T T G T T C C A T A C A T G G T T A G A T A G A C A T A G A G A T A T G T G C G A A A A G T G G G A T A C A A C A A T A A A A A G A G A A T T G T T A G 9048

N L F H T W L D R H R D M C E K W D T N N K K E E L L 2774

A T A A A T T A A A A G A A T G G A A T A A A G A T A A C A A T A G T G G T A A T A T T A A C C C T A G T G G T A A T A C C C C A C C A C T A G T G A C A 9129

D K L K E E W N K D N N S G N I N P S G N T P P T S D 2801

T A C C T A G T G T A A C A A A G T G A T A T A C C T A G T G A T A A C A C A T A C A T A G T G A C A T A C C A T A C G T G T T A A A T A C T G A C G T T T 9210

I P S G K Q S D I P S D N N I H S D I P Y V L N T D V 2828

C T A T A C A A A T A C A T A T G G A T A A T C C T A A A C C T A A A C G A A T T T C A A A T A T G G A T A C A T A C C C T A A T A T T C T T C C A T G G 9291

S I Q I H M D N P K P I N E F S N M D T Y P N N S S M 2855

FIG. 12-23

SUBSTITUTE SHEET (RULE 26)

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A T A C T A T C T T G G A G G A T C T G G A C A A A C C A T T T A A T G A A C C C T A C T A T T A T G A T G T G C A A G A T G A T A T T T A T T A T G A T G T A C 9372

D T I L E D L D K P F N E P Y Y Y D V Q D D I Y Y D V 2882

A T G A T C A T G A T A C A A C T G T G G A T A C T A A T G C T A T G G A T G A A C C T A G C A A A G T A C A A A T T G A A A T G G A T G T A A A T A C G A 9453

H D H D T S T V D T N A M D E P S K V Q I E M D V N T 2909

A A T T G G T G A A G A A A T A T C C T A T A G C A G A T T T A T G G A T A T A 9497

K I V K E K Y P I A D L W D I 2924

*FIG. 12-24.*

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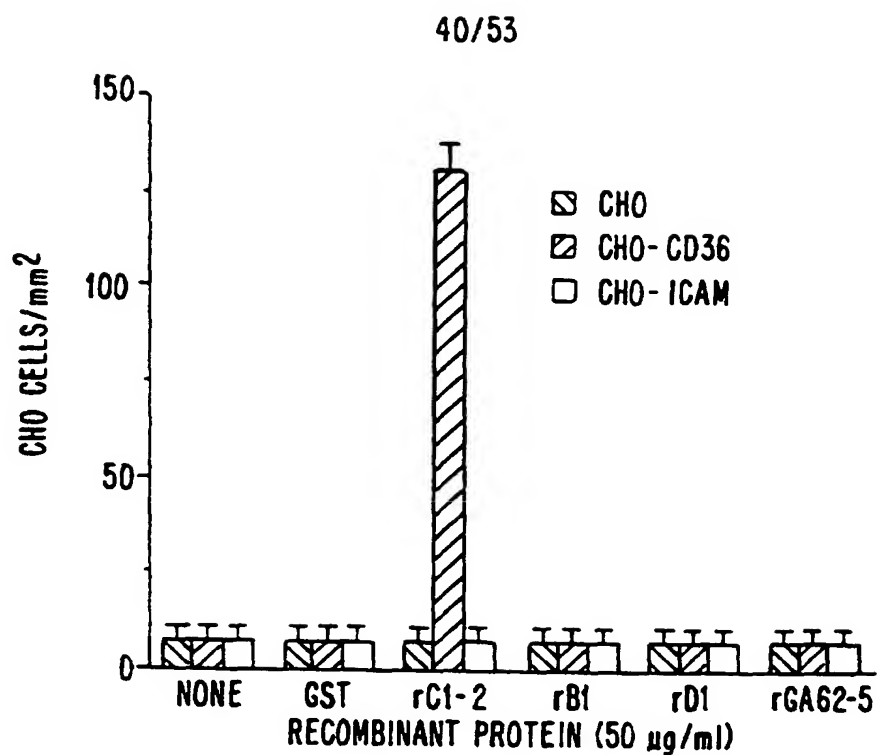


FIG. 13.

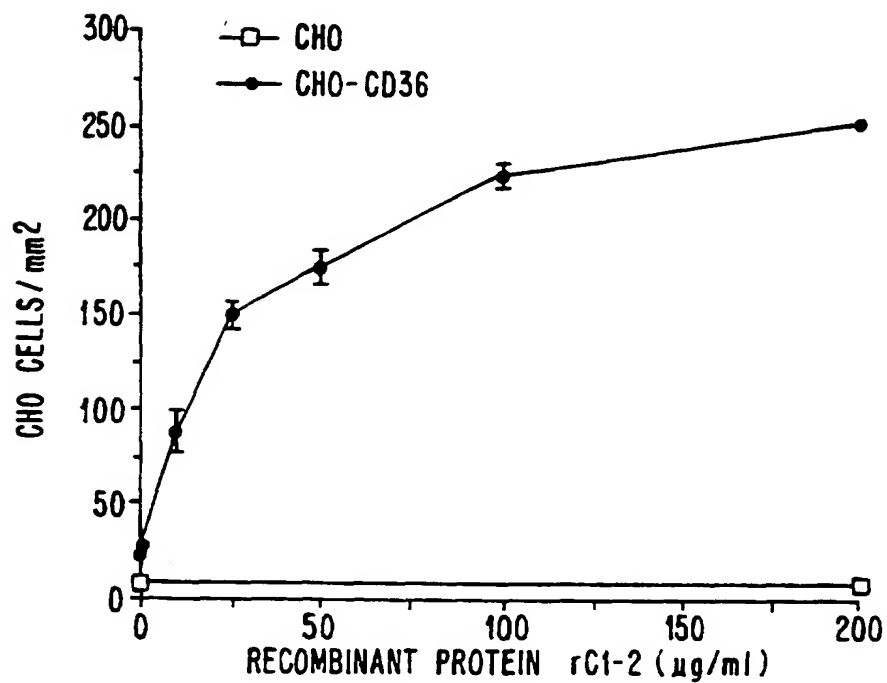
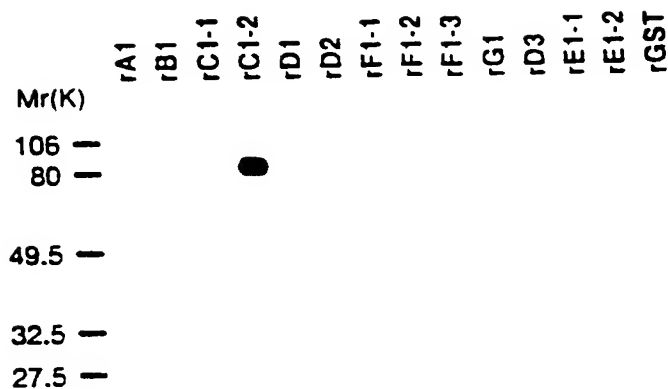
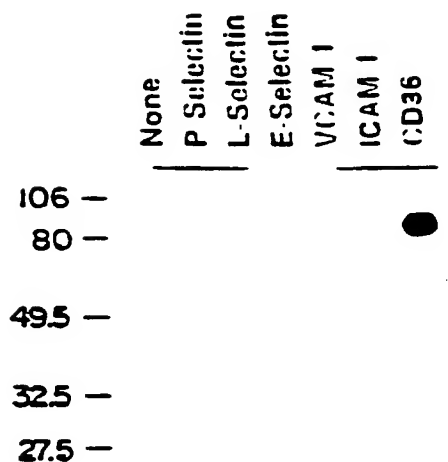


FIG. 14.

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*FIG. 15A.*

Binding of CD36 to  
Immobilized rC1-2

*FIG. 15B.*

SUBSTITUTE SHEET (RULE 26)

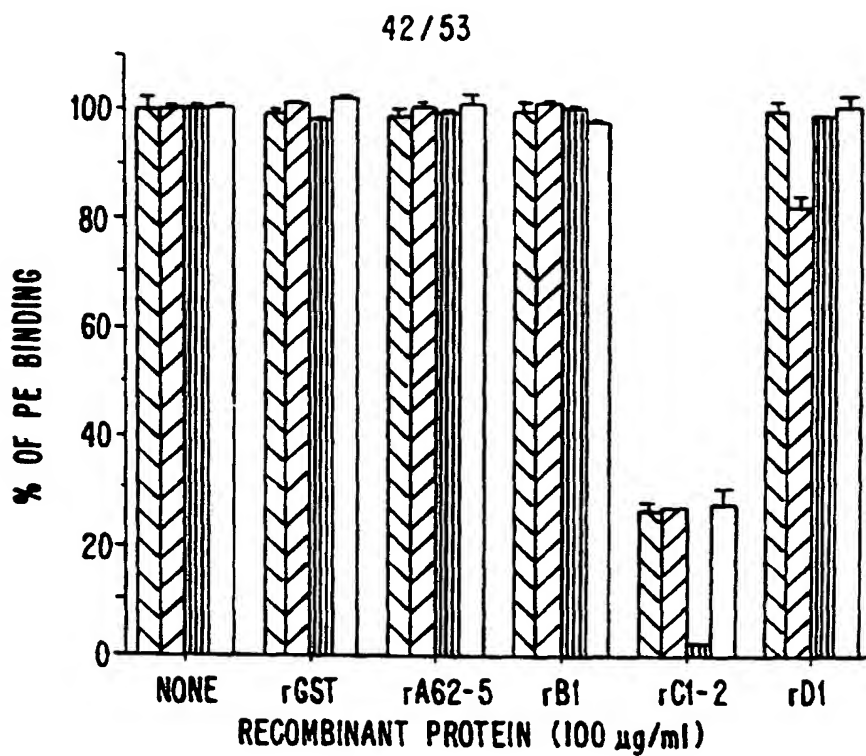


FIG. 16.

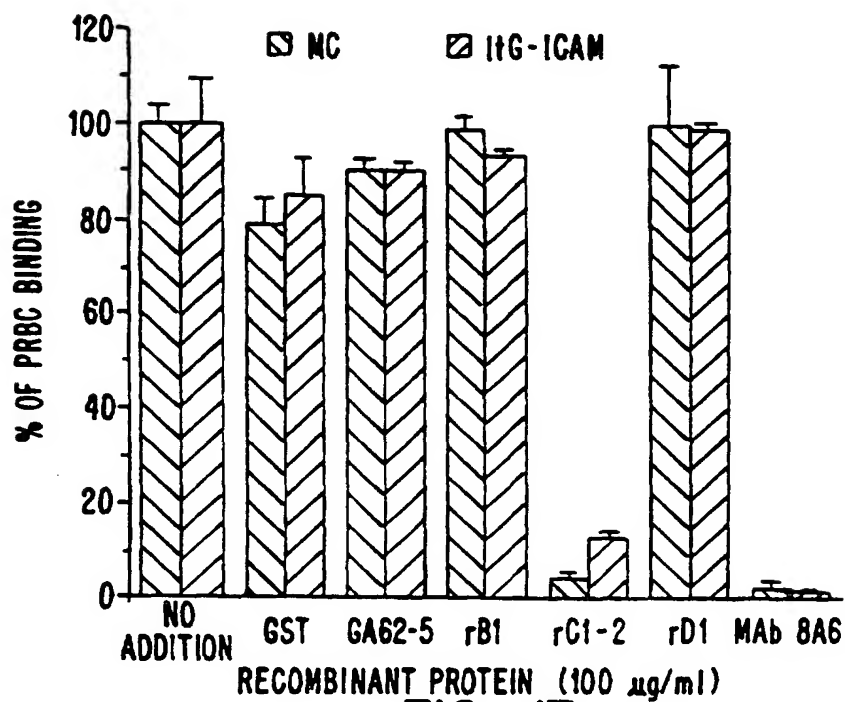


FIG. 17.

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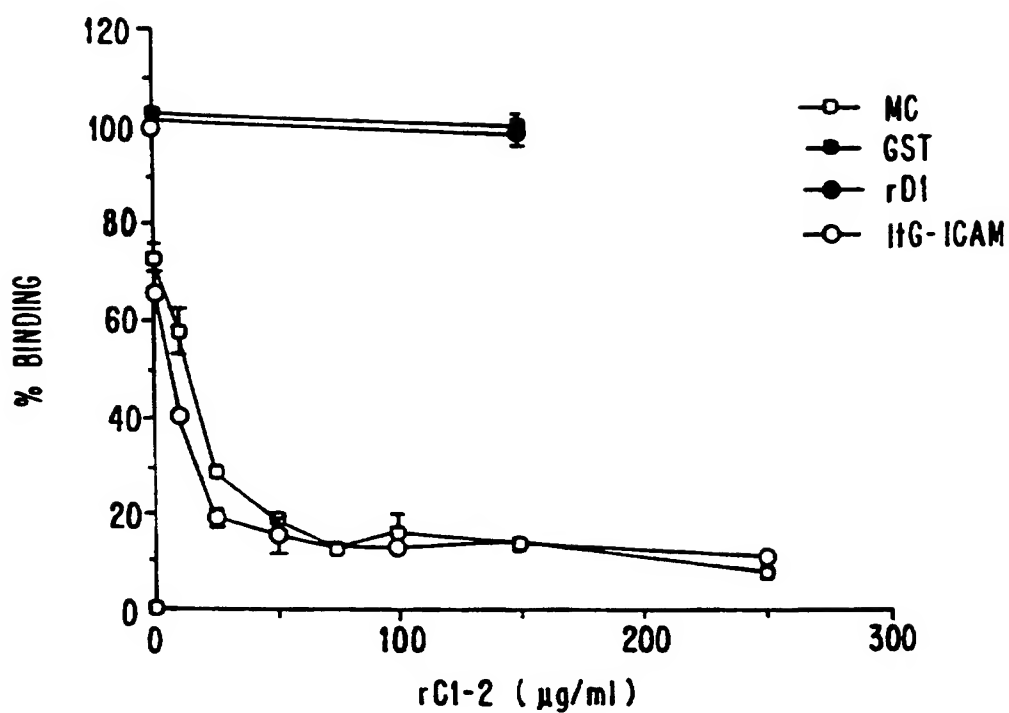
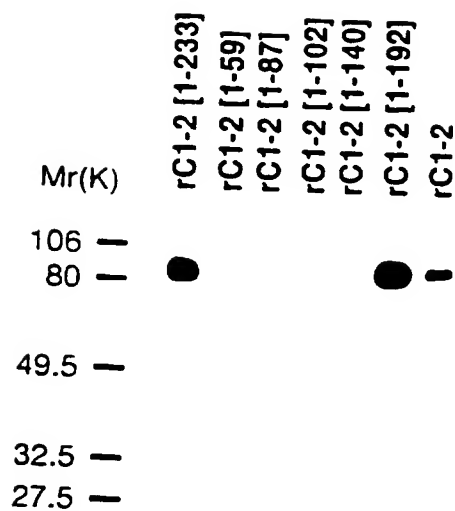


FIG. 18.

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**FIG. 19.**



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	70	80	90	100	110	120	130	140	150		
	G	L	MK	L	Q	DE	D	ER	E	IME	I
CC	F	KQKDIP	F	T	VLEG	VLE	I	YGNA	EIKHIK	LLD	GGG
(1)	FRKQKDIPKDWTHDDFLQT---	LLMKDLLLEI	---	IQD	TYGDANEIKRIE	ALL	EAQ	---	VGGIDFAALAGLYTKGF		
(2)	FRKQKDIPKDWTHDDFLQT---	LLMKDLLLEI	---	IQD	TYGDANEIKRIE	ALL	EAQ	---	VGGIDFAALAGLYTKGF		
(3)	FRKQKDIPKDWTHDDFLQT---	LLMKDLLLEI	---	IQD	TYGDANEIKRIE	ALL	EAQ	---	VGGIDFAALAGLYTKGF		
(4)	FRKQKDIPKDWTHDDFLQT---	LLMKDLLLEI	---	IQD	TYGDANEIKRIE	ALL	EAQ	---	VGGIDFAALAGLYTKGF		
(5)	FKKQKDIQKGVPIVF-	THDDVLEG--	VLSGELFKNIEKDYGNAE	EIKHIKEIMDNKNRQETTGGGTQ	---						
(6)	IKKQKDIQKGVPIVF-	THDDVLEG--	VLSGELFKNIEKDYGNAE	EIKHIKEIMDNKNRQETTGGGTQ	---						
(7)	FKKQKDIQKGVPIVF--	THDDVLEG--	VLSGELFKNIEKDYGNAE	EIKHIKEIMDNKNRQETTGGGTQ	---						
(8)	FKKQKDIQKGVPIVF--	THDDVLEG--	VLSGELFKNIEKDYGNAE	EIKHIKEIMDNKNRQETTGGGTQ	---						
(9)	FKKQKDIQKGVPIVF--	THDDVLEG--	VLSGELFKNIEKDYGNAE	EIKHIKEIMDNKNRQETTGGGTQ	---						
(10)	FKKQKDIQKGVPIVF--	THDDVLEG--	VLSGELFKNIEKDYGNAE	EIKHIKEIMDNKNRQETTGGGTQ	---						
(11)	FKRQKDIQKGVPIVF--	THDDVLEG--	VLSGELFKNIEKDYGNAE	EIKHIKEIMDNKNRQETTGGGTQ	---						
(12)	FDKQKDIPD-GRY--	FL-TLEGVLEG--	VLEKGVLLTSIKEGYGNERDIEHIKQLLDEEEAAGALGGGG--	AALGGGLYTHGP							
(13)	FDKQKDIPD-GRY--	FL-TLEGVLEG--	VLEEGVLLTSIKEGYGNERDIEHIKQLLDEEEAAGALGGGG--	AALGGGLYTHXA							
(14)	FDKQKDIPD-GRY--	FL-TLEGVLEG--	VLEEGVLLTSIKEGYGNERDIEHIKQLLDEEEAAGALGGGG--	AALGGGLYTHXA							

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FIG. 20-2.

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	160	170	180	190
	NK	I N	E	
C	KD	TTIDKLLQHE	KC	D C PP RSVAR
(1)	VAE-KD	TTIDKLLQHEQK	EADKCLKTH	DDTC----PPQEDRSVAR
(2)	VAE-KD	TTIDKLLQHEQK	EADKCLKTH	DDTC----PPQEDRSVAR
(3)	VAE-KD	TTIDKLLQHEQK	EADKCLKTH	DDTC----PPQEDRSVAR
(4)	VAE-KD	TTIDKLLQHEQK	EADKCLKTH	DDTC----PPQEDRSVAR
(5)	----	NKTTIDKLLNHEEG	IAKECQKQ	KN-D-CNKQSPPT-TDRSVAR
(6)	----	NKTTIDKLLNHEEG	IAKECQKQ	KN-D-CNKQSPPT-TDRSVAR
(7)	----	NKTTIDKLLNHEEG	IAKECQKQ	KN-D-CNKQSPPT-DEVSPA
(8)	----	NKTTIDKLLNHEEG	IAKECQKQ	KN-D-CNKQSPPT-TDRSVAR
(9)	----	NKTTIDKLLNHEEG	IAKECQKQ	KN-D-CNKQSPPT-TDRSVAR
(10)	----	NKTTIDKLLNHEEG	IAKECQKQ	KN-D-CNKQSPPT-TDRSVAR
(11)	----	NKTTIDKLLNHEEG	IVKECQKQ	KN-D-CNKQSPPT-TDRSVAP
(12)	VAGQ-D	TTIDKILQHEDK	DATKC--K-N	---C-K--PP-EDRSVAR
(13)	VAGQ-D	TTIDKILQHEDK	DATKC--K-N	---C-K--PP-EDRSVAR
(14)	VAGQ-D	TTIDKILQHEDK	DATKC--K-N	---C-K--PP-EDRSVAR

FIG. 20-3.

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	10	20	30	40	50	60																												
	H	I	D	K	Q	L	K	K	C	I	C	N	K	C	F	K	W	V	E	K	K	T	E	W	G	K								
FW	WV	D	M	L	H	D	S	*E	W	R	E	H	C	I	N	N	K	C	I	K	C	F	K	W	V	E	K	K	T	E	W	G	K	
CONSENSUS																																		
(1)	CMC	uni#1																																
(2)	CMC	uni#2																																
(3)	CMC	uni#3																																
(4)	CMC	uni#4																																
(5)	gMC	K- uni#1																																
(6)	gMC	uni#5																																
(7)	gMC	uni#6																																
(8)	gMC	K+ uni#1																																
(9)	CMC	uni#7																																
(10)	gC5	uni#1																																
(11)	gHB3	uni#1																																
(12)	gPA	uni#1																																
(13)	gDd2	uni#1																																

FIG. 21-1.

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	70	80	90	100	110	120	130	140	
	V	V D V	D	LQE HANTNE	K	V	D		
C	IK HF KQ DI	TLE*LL	G LL	IKD YGDADDIKHIK	LL EE	A G	A GG		
(1)	IKDHF	KQKDI	PKDWT---	H---	DDFLQ	LLMKD	LLLEII	QDITYGDANEIKRIEALLEQ---AGVGGIDFAALAGLYTKG	
(2)	IKEHF	DKQDI	PIYE-C---	Y---	FTTLE	GVLEK	GVLLT	SLQEAYGNANEIKHIKELLEKEEADG--GDGAAAFGDPCTKV	
(3)	IKEHF	DKQDI	PIYE-C---	Y---	FTTLE	GVLEK	GVLLT	SLQEAYGNANEIKHIKELLEKEEADG--GDGAAAFGDPCTKV	
(4)	IKEHR	DKQDI	PIYE-C---	Y---	FTTLE	GVLEK	GVLLT	SLQEAYGNANEIKHIKELLEKEEADG--GDGAAAFGDPCTKV	
(5)	IKEHF	DKQDI	PIYE-C---	Y---	FTTLE	GVLEK	GVLLT	SLQEAYGNANEIKHIKELLEKEEADG--GDGAAAFGDPCTKV	
(6)	IVKHF	YKQEDIG	QKEVPI	VFTHDY	VLEGVLEK	GVLLT	SIKDVHGD	TDDIKHIKDLLNEEEAAVAG-----ASGG-----	
(7)	IVKHF	YKQEDIG	QKEVPI	VFTHDY	VLEGVLEK	GVLLT	SIKDVHGD	TDDIKHIKDLLNEEEAAVAG-----ASGG-----	
(8)	IVKHF	YKQEDIG	QKEVPI	VFTHDY	VLEGVLEK	GVLLT	SIKDVHGD	TDDIKHIKDLLNEEEAAVAG-----ASGG-----	
(9)	IKKHF	IKQDDIG	QETN---	CDPM	VTLEI	LLD	IDELL	KNIKDTHANADDID	
(10)	IKKHF	IKQDDIG	QETN---	CDPM	VTLEI	LLD	IDELL	KNIKDTHANADDID	
(11)	IKEHF	DKQDI	PDG-R---	Y---	FLTLE	GVLEK	GVLLT	SIKEGYGNEKDIEHIKQLLDEEEAAGALGGGAALGGLYTQG	
(12)	IKDH	FGKQTD	IVQQKGLI	VFSPY	GVLD	VLK	GGNLLQ	NIKDVHGD	TDDIKHIKLLDEEDA-VAVV-----LGG-----
(13)	IKDH	FGKQTD	IVQQKGLI	VFSPY	GVLD	VLK	GGNLLQ	NIKDVHGD	TDDIKHIKLLDEEDA-VAVV-----LGG-----

FIG. 21-2.

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	150	160	170	180
	END			
	KDNTTIDK L H			
(1)	FVAEKD	TTIDKILQH		
(2)	G-DKDN	TTIDKMLKH		
(3)	G-DKDN	TTIDKILNH		
(4)	G-DKDN	TTIDKCLQH		
(5)	G-DKDN	TTIDKLLNH		
(6)	---	ENNTTIDKMLKH		
(7)	---	ENNTTIDKMLQH		
(8)	---	ENNTTIDKMLKH		
(9)				
(10)				
(11)	PVAGQD	TTIDKLLQH		
(12)	---	KDNTTIDKMLNH		
(13)	---	KDNTTIDKLLQH		

FIG. 21-3.

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MC PIEMPI

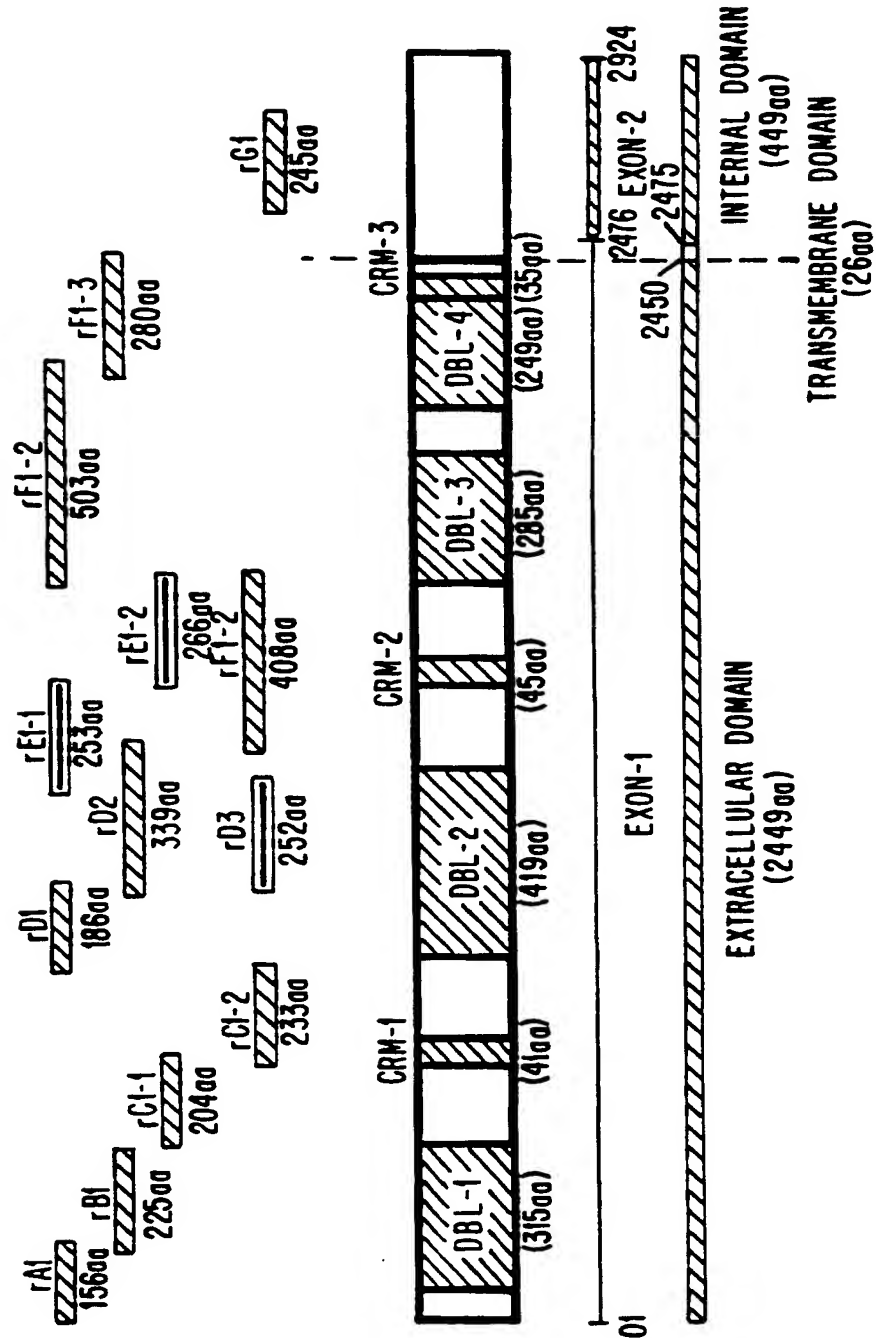
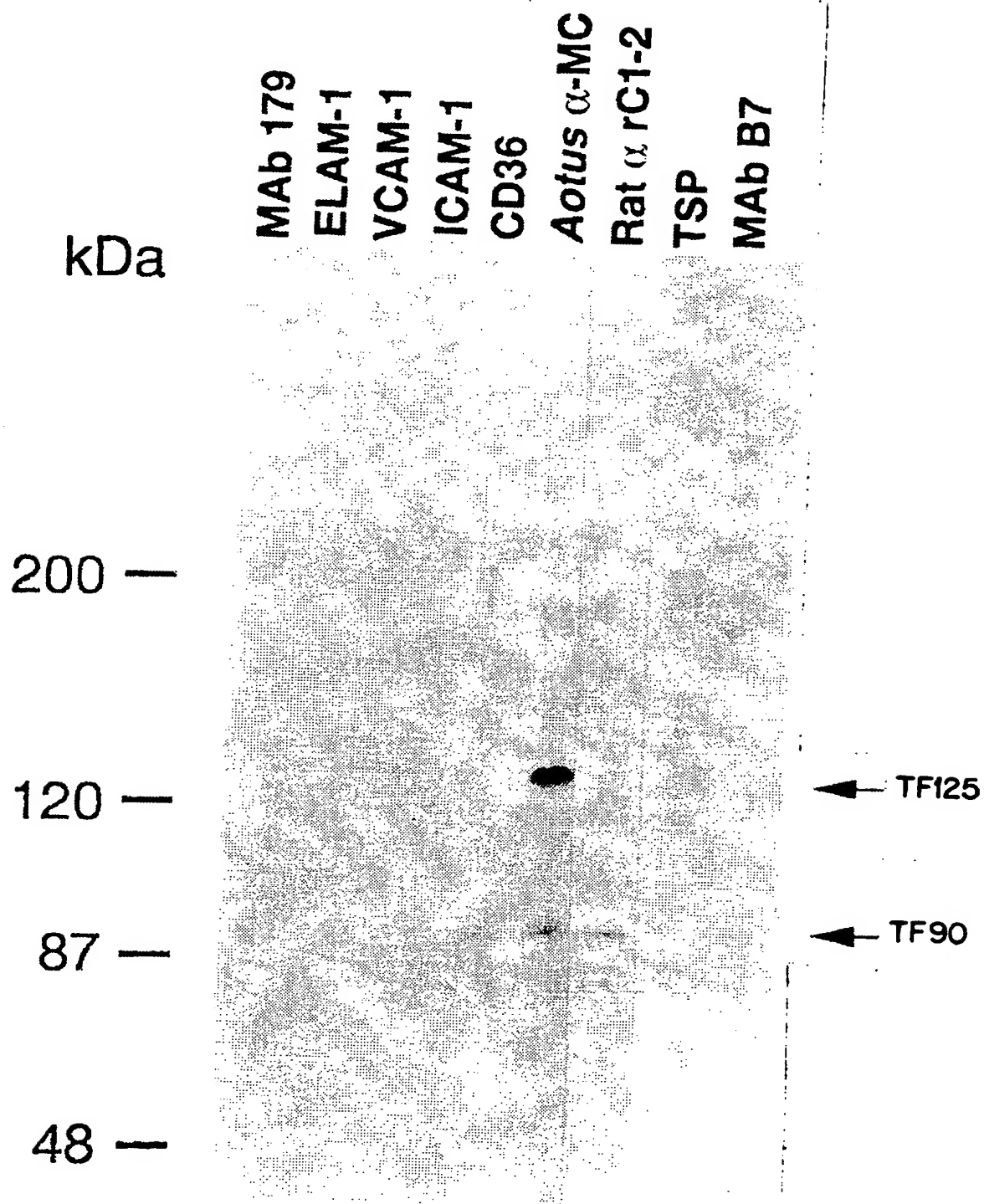


FIG. 22.

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**FIG. 23.**

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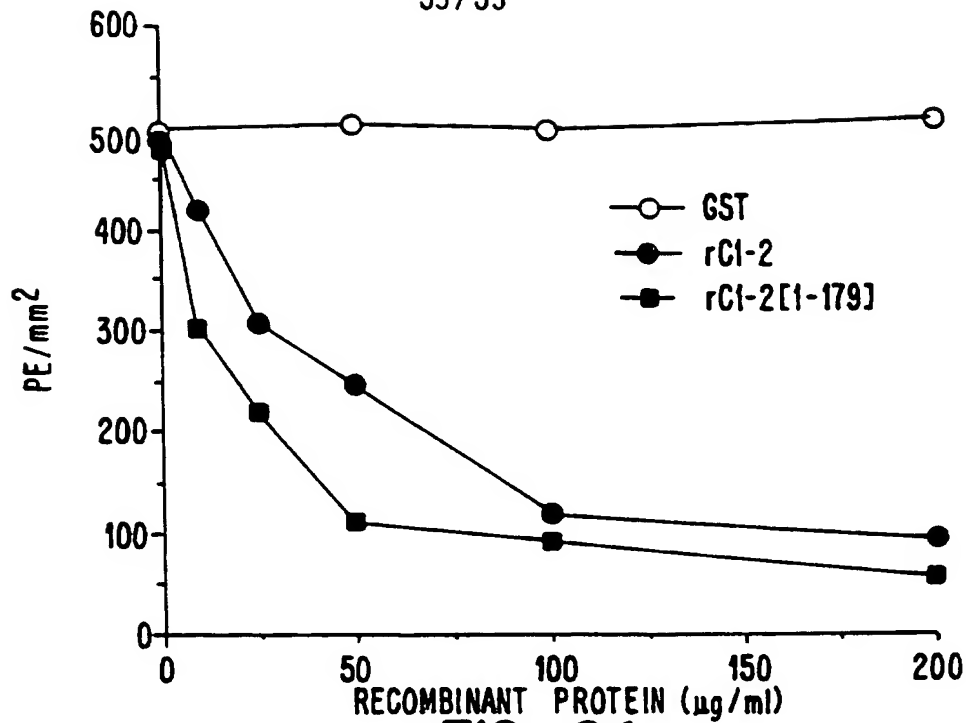


FIG. 24.

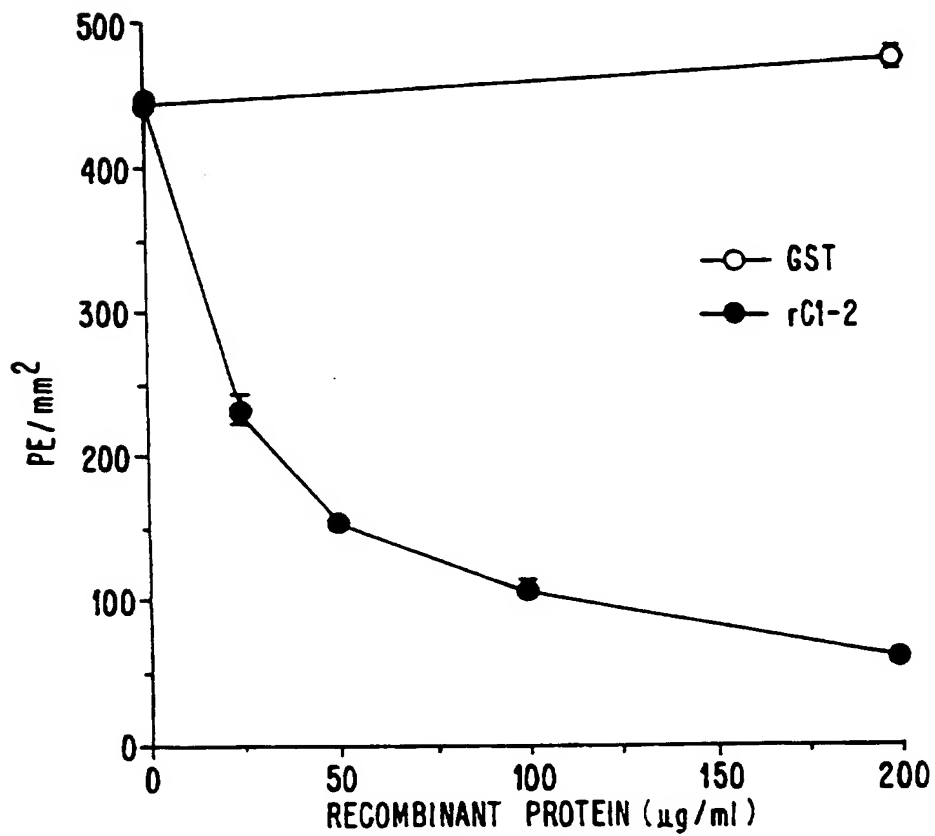
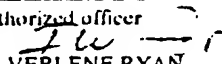


FIG. 25.

SUBSTITUTE SHEET (RULE 26)

# INTERNATIONAL SEARCH REPORT

National application No.  
PCT/US96/05798

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(5) : A61K 39/015; C12P 21/02 US CL : 424/191.1, 272.1; 435/258.2 According to International Patent Classification (IPC) or to both national classification and IPC				
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 424/191.1, 272.1; 435/258.2  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, CAPLUS, MEDLINE, BIOSIS, EMBASE, SCISEARCH, WPIDS				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X --- Y	PASLOSKE, B.L. Annu. Rev. Med. "MALARIA THE RED CELL, AND THE ENDOTHELIUM", 05 April 1994, Vol. 45, pages 283-295, especially pages 283, 290-292.	1-9, 33 ----- 39-41, 45-47		
A	LEECH, Plasmodium falciparum cytoadherence. Res. Immunol. 1991, Vol. 142, No. 8, pages 681-686.	1-9, 33, 39-41, 45-47		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;">           * Special categories of cited documents:            "A" document defining the general state of the art which is not considered to be part of particular relevance            "E" earlier document published on or after the international filing date            "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)            "O" document referring to an oral disclosure, use, exhibition or other means            "P" document published prior to the international filing date but later than the priority date claimed         </td> <td style="width: 50%; border: none;">           "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention            "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone            "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art            "Z" document member of the same patent family         </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be part of particular relevance "E" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be part of particular relevance "E" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family			
Date of the actual completion of the international search  06 AUGUST 1996		Date of mailing of the international search report  05 SEP 1996		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer  VERLENE RYAN Telephone No. (703) 308-0196		

Form PCT/ISA/210 (second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/05798

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOWARD, Two approximately 300 kilodalton Plasmodium falciparum proteins at the surface membrane of infected erythrocytes. Molecular and Biochemical Parasitology, 1988, Vol. 27, pages 207-224.	1-9, 33
Y	WO 94/03604 A1 (SCHERING CORPORATION) 17 February 1994, see entire document, especially pages 42-44.	33, 39-41, 45-47

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/05798

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-9, 33, 39-41, 45-47

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)\*

**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-9, 33, 39-41, and 45-47, drawn to a polypeptide comprising PIEMP1 and a method of using the polypeptide to prevent infection in a patient.

Group II, claims 10-26, and 34, drawn to nucleic acid

Group III, claims 42-44, drawn to a method of treating a patient by using the nucleic acid.

Group IV, claims 53, drawn to a method of identifying *Plasmodium falciparum* parasite by using the nucleic acid.

Group V, claims 27-32, 35, drawn to an antibody.

Group VI, claims 48-50, drawn to a method of using the antibody to prevent infection, and a method of using the antibody to prevent infection.

Group VII, claims 36-38, 51-52, drawn to diagnostic applications using PIEMP1.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Although the three distinct products, i.e. the polypeptide, the nucleic acid, and the antibody are related via their individual relationship to the PIEMP1 protein, the PIEMP1 protein is not present in all the claims of each group. For example, the polypeptide of Group I is derived from PIEMP1 protein, however, the polypeptide can be prepared using recombinant or synthetic methods. In addition, the antibodies of Group V can be produced by the nucleic acid or the polypeptide, and they can be used for probes, or in assays to detect particular antigens.

Since the groups do not have a common special technical feature, unity of invention is lacking.

The inventions listed as Group I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the seven groups are related by their relationship to the PIEMP1 polypeptide.